STUDIES ON THE BIOAVAILABILITY OF ACTIVE PRINCIPLES FROM PROCESSED SPICES

THESIS

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In Biochemistry

By
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DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON THE BIOAVAILABILITY OF ACTIVE PRINCIPLES FROM PROCESSED SPICES"** submitted to the University of Mysore for the award of degree of DOCTOR OF PHILOSOPHY is the result of the research work carried out by me in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India under the guidance of **Dr. K. Srinivasan** during the period of 2001 – 2006.

I further declare that the research work embodied in this thesis has not been submitted for the award of any other degree.

(D. Suresh)
7th August 2006

Dr. K. Srinivasan,
Senior Scientist,
Department of Biochemistry and Nutrition.

CERTIFICATE

I hereby certify that the thesis entitled **"STUDIES ON THE BIOAVAILABILITY OF ACTIVE PRINCIPLES FROM PROCESSED SPICES"** submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY by **Mr. D. SURESH** is the result of the research work carried by him in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore under my guidance during the period of 2001 – 2006.

K. Srinivasan
(Guide)
7th August 2006

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List of abbreviations

Å	Angstrom
ANS	8-Anilino-Naphthalene sulfonic acid
BSA	Bovine serum albumin
Cal	calorie
CD	Circular Dichroism
ºC	degree Celsius
cm	centi meter
FRET	Fluorescence resonance energy transfer
G	gram
X g	times gravity
h	hour
HSA	Human serum albumin
K_b	Association constant (M^{-1})
k cal	kilocalorie
k Da	kilo Dalton
kJ	kilo Joule
M	molar concentration
mg	milligram
min	minutes
μl	micro liter
μg	micro gram
mol	moles
MRW	mean residual weight
n	total number of binding sites
n mol	nano moles
nm	nano meter
NADP	β -Nicotinamide adenine dinucleotide phosphate
NADPH	β -Nicotinamide adenine dinucleotide phosphate - reduced
NAD	β -Nicotinamide adenine dinucleotide
NADH	β -Nicotinamide adenine dinucleotide – reduced

PAGE	Poly acrylamide gel electrophorersis
R	Universal gas constant
rpm	rotations per minute
sec	second
SEM	Standard error mean
SDS	sodium dodecyl sulphate
T	absolute temperature
TIB	Tri Iodo Benzoic Acid
Tris	Tris (hydroxy methyl) amino methane
UV	ultra voilet
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
$E_{1\%}$	Absorption coefficient of 1% protein solution
ΔA	Change in absorbance
$\in$	molar extinction coefficient ($M^{-1}cm^{-1}$)
$\Delta \in$	change in molar extinction coefficient
ΔG	Gibb`s free energy change
ΔH	Enthalpy change
ΔS	Entropy change

CONTENTS

List of Tables

Chapter - 2

Chapter - 3

Chapter - 4

Chapter - 5

List of Figures

Chapter – 1

Chapter – 2

Chapter – 4

CHAPTER - I

GENERAL INTRODUCTION

1. Spices

Spices are a group of esoteric food adjuncts that have been in use for thousands of years to enhance the sensory quality of foods. The quantity and variety of spices consumed in tropical countries is particularly extensive. These spice ingredients impart characteristic flavor, aroma, or piquancy and color to foods. Apart from these qualities, a host of beneficial physiological influences are attributed to the spices. Among these, beneficial influence on lipid metabolism, efficacy as anti-diabetics, ability to stimulate digestion, antioxidant property and anti-inflammatory potential are extensively studied.

Digestive stimulant action: Spices are well recognized to stimulate gastric function. They are generally believed to intensify salivary flow and gastric juice secretion and hence aid in digestion (Glatzel, 1968). Earlier reports on the digestive stimulant action of spices are largely empirical; it is only in recent years that this beneficial attribute of spices has been authenticated in exhaustive animal studies (Platel & Srinivasan, 2004). Animal studies have revealed that a good number of spices, when consumed through diet, bring about an enhanced secretion of bile with higher bile acid content, which plays a vital role in fat digestion and absorption (Bhat *et al*, 1985). Spices that stimulate bile acid production by liver and its secretion in to the bile include turmeric, red pepper, ginger, cumin, coriander, ajowan, fenugreek, mustard, onion and tamarind.

Hypolipidemic property: Some of the spices are evaluated for a possible hypocholestrolemic action in a variety of experimental situations in both laboratory animals and humans. The spices - fenugreek, red pepper, turmeric, garlic, and onion were found to be effective as hypocholestrolemic agents under various conditions of experimentally induced hypercholesterolemia or hyper-lipidemia. Further fenugreek,

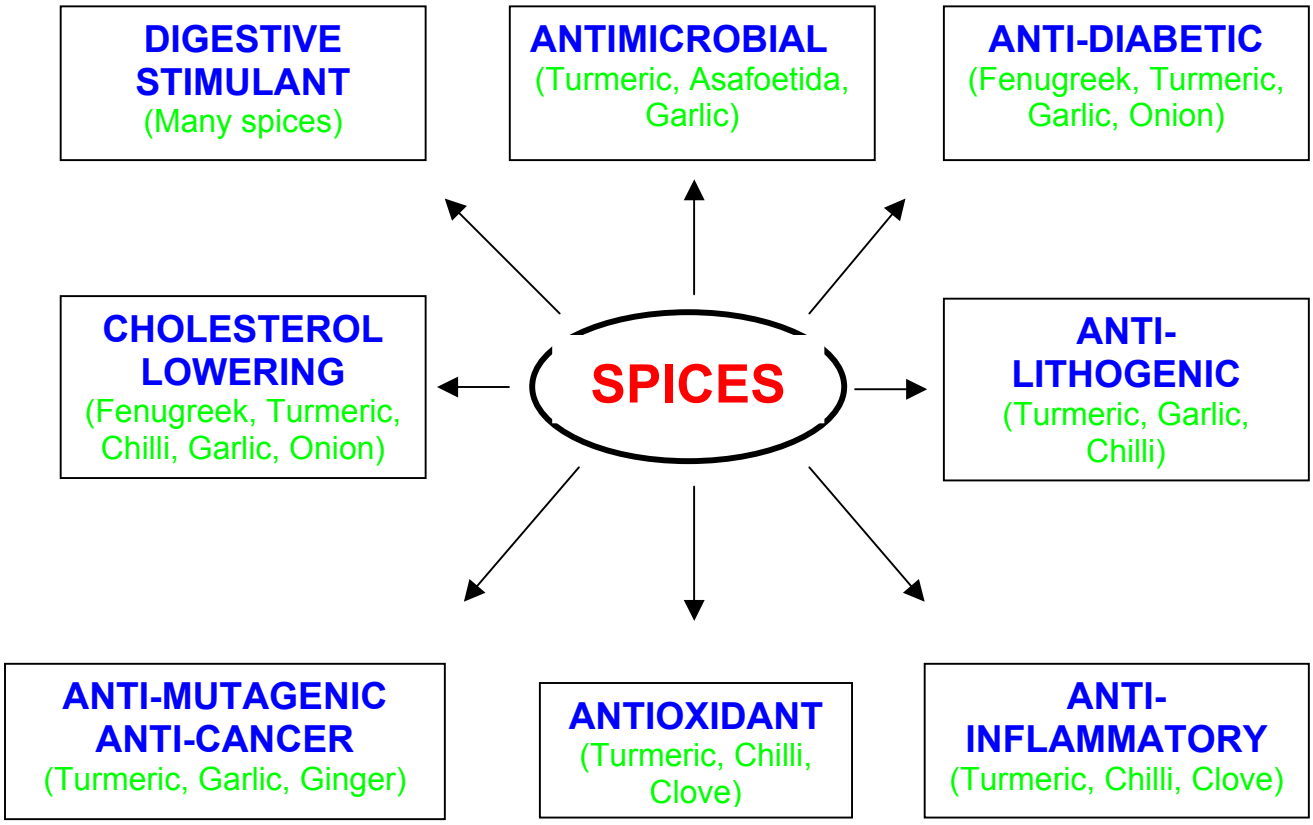


Fig. 1 Summary of physiological effects of spices.

onion and garlic are effective in humans with hypelipidemic condition (Srinivasan *et al*, 2004). Fenugreek seeds were hypocholesterolemic in rats with hyperlipidemia induced by either high fat diet (Singhal *et al*, 1982) or high cholesterol diet (Sharma, 1984). Turmeric (Srimal, 1987) red pepper (Suzuki & Iwai, 1984), onion and garlic (Fenwick & Hanley, 1985; Carson, 1987) have been proved to be hypolipidemic.

Antidiabetic property*:* Fenugreek, garlic, onion, turmeric and cumin have been studied for their antidiabetic potential. Such studies have unequivocally demonstrated that antidiabetic potential of fenugreek in both type-I and type-II diabetes. Addition of fenugreek seeds to the diets of diabetic patients or animals results in a significant fall in blood glucose and improvement in glucose tolerance (Sharma *et al*, 1996; Khosla *et al*, 1995*).* Garlic and onion have been widely studied for their antidiabetic potential. Both these spices were shown to be hypoglycemic in different diabetic animal models and in limited human trials. The hypoglycemic potency of these spices is attributed to the sulfur compounds present in them (Kumudkumari *et al*, 1995; Augusti & Sheela, 1996). Hypercholesterolemia is a regular biochemical abnormality in diabetes mellitus and nephropathy is one of the secondary eventualities of this disease. Extensive studies with diabetic rat models have been carried out to examine whether with hypolipidemic spices supplementation in the diet would influence renal lesions associated with diabetes by virtue of their possible cholesterol lowering effect. Dietary curcumin and onion have been found to have a promising ameliorating influence on the severity of renal lesions in streptozotocin induced diabetic rats (Babu & Srinivasan, 1999).

Antioxidant property*:* Oxidative damage at the cellular and sub-cellular level is now considered to be an important event in disease processes like CVD, inflammatory disease, carcinogenesis, and ageing. Dietary curcumin and onion were shown to

effectively lower the level of lipid peroxides in circulation and the amount excreted in diabetic animals (Babu & Srinivasan, 1997). Spice principles – curcumin and capsaicin and the spice garlic were evidenced to bring about beneficial restoration of the altered antioxidant status in the erythrocytes in hypercholesterolemic and in hyperlipidemic rats (Kempaiah & Srinivasan, 2004, 2004a). Among the other spices examined for their antioxidant properties, black cumin extracts showed potent inhibitory activity on lipoxygenases and on oxidation of LDL-lipids. Search for the identification of antioxidant principle in black cumin narrowed it down to the presence of tannic acid.

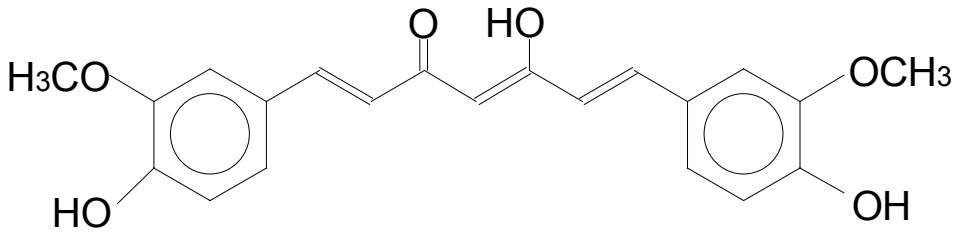
Antiinflammatory property: Turmeric happens to be the earliest anti-inflammatory drug known in the indigenous system of medicine in India. Turmeric extract, curcuminoids and volatile oil of turmeric have been found to be effective in experiments with mice, rats, rabbits and pigeons. The efficacy of curcuminoids was also established in carrageenan-induced foot paw edema in mice and rats and in cotton pellet granuloma pouch tests in rats; in the latter, curcumin was comparable to phenyl butazone (Srimal, 1997). The anti-inflammatory effect of curcumin in patients who had undergone surgery for hernia / hydrocele was found comparable to that of phenyl butazone (Satoskar *et al*, 1986).

Chemopreventive property: The mustard extract has the property of inactivating the mutagenicity of food mutagens like tryptophan pyrolysate. The antimutagenic effects of mustard seed powder have been assed in experimental animals treated with potent carcinogens. These experiments suggest that mustard has excellent antimutagenic property. The antimutagenic effects of curcumin have been most widely studied (Anto *et al*, 1996; Surh, 1999) and this has been shown to be antimutagenic in several experimental systems. Recently, considerable attention has been focused on

identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing the multi-stage carcinogenesis. The anticancer potential of curcumin as evidenced by both preclinical and clinical studies has been exhaustively reviewed very recently (Aggarwal *et al*, 2003). Several studies indicate that curcumin can suppress both tumour initiation and tumour promotion. Curcumin has been demonstrated to have anti-tumour effect in animals treated with potent carcinogens. Garlic is yet another spice widely studied in recent years for its chemopreventive potential. Epidemiological studies have shown that higher intake of *allium* products is associated with reduced risk of several types of cancers, especially stomach and colorectal (Fleischauer & Arab, 2001). These epidemiological findings are well correlated with several laboratory investigations. Several mechanisms have been proposed to explain cancer preventive effects of widely used garlic and its organosulfur compounds (Sengupta *et al*, 2004). Pungent vanilloids especially [6]-gingerol present in the widely used dietary spice - ginger (*Zingiber officinale*) have been found to possess potential chemo-preventive activities. Prior topical application of [6]-gingerol or [6]-paradol significantly suppressed the tumor promoter (phorbol ester) stimulated skin inflammation initiated by 7,12-dimethylbenz [α] anthracene in mice (Surh *et al*, 1999).

2. Spice active principles: Curcumin, Capsaicin and Piperine

2.1 Curcumin



Curcumin (Diferuloyl methane), the natural yellow pigment in turmeric, is isolated form rhizomes of the plant *curcuma longa*. It constitutes about 3 - 4 % of the rhizome of turmeric. With the discovery that the dried rhizome of *curcuma longa* is very rich in polyphenols, the spice turmeric became vital in medicine. These polyphenols are known to possess antioxidant properties. Free radical mediated damage to biological systems is recognized as the initiating agent for many diseases such as cardiovascular diseases, cancer and arthritis. Turmeric and its constituent curcumin show beneficial effects on these diseases and on other illness (Eigner & Scholz, 1999). For example, the low incidence of large bowel cancers in Indians could be attributed to a high intake of natural antioxidants, such as curcumin in the diet (Mohandas & Desai, 1999). Numerous research teams provided evidence that curcumin contributes to the inhibition of tumour formation and promotion as cancer initiation, promotion or progression of tumours is decreased or blocked by this compound. Azuine and Bhide (1992) described curcumin as an inhibitor of tumour formation and promotion induced by benz(α)pyrene, 7,12-dimethyl-benz(α)anthracene or phorbol esters, while Ikezaki *et al*. (2001) demonstrated that curcumin inhibits cancer development in rat stomach initiated by *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine (MNNG). In the same way, bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione, a bis-demethoxy-curcumin analog (BDMC-A), blocks the formation of colon adenocarcinoma in rats (Devasena *et al*, 2003). Although curcumin does not decrease the copper-induced liver or kidney tumour incidence in Long-Evans Cinnamon (LEC) rats, an inbred mutant strain which accumulates copper due to an aberrant copper-transporting ATPase gene, it reduces overall cancer formation as well as formation of metastasis (Frank *et al*, 2003). Furthermore, curcumin was described as a good anti-angiogenesis agent (Arbiser *et al*, 1998), explaining its chemopreventive effect at the level of tumour promotion. This

phenomenon could be explained by vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 inhibition in EAT cells, by VEGF and angiopoietin-1 inhibition in NIH 3T3 cells and by inhibition of the tyrosine kinase Flk-1/KDR (VEGF receptor-2) in HUVEC cells (Gururaj *et al*, 2002). Concerning clinical trials, curcumin was given to patients with early stages of cancer up to 12 g/day (Cheng *et al*, 2001). This treatment did not present cytotoxicity up to 8 g/day, however beyond 8 g/day, the bulky volume of the drug became unacceptable to patients. Anti-cancer effect of curcumin seems to be potentialized in the presence of oestrogen in breast cancer cells and it inhibits genes which are under the influence of the oestrogen receptor (Shao *et al*, 2002). Curcumin also displays an inhibiting effect on human telomerase reverse transcriptase (hTERT) expression, reducing telomerase activity in MCF-7 cells (Ramachandran *et al*, 2002). Moreover, it allows sensitizing ovarian cancer cells to cisplatin, enhancing chemotherapeutic treatment (Chan *et al*, 2003).

It has been understood that curcumin inhibits cyclooxygenase-2 (COX-2) as well as lipoxygenase (LOX), two enzymes involved in inflammation (Huang *et al*, 1991). Indeed, cytokine-induced COX-2 transforms arachidonic acid in prostaglandins during acute inflammatory episodes. COX2 is also the prevalent isoform during chronic inflammations. Lipoxygenase transforms arachidonic acid in leukotrienes, which take part in leukocytes recruiting and play a role in inflammation (Fiorucci *et al*, 2001).

Curcumin protects keratinocytes and fibroblasts against H_2O_2-induced damages (Phan *et al*, 2001) and allows reduction of oxidative and inflammatory stress in Alzheimer patients (Lim *et al*, 2001). Pancreatitis improves after curcumin treatment, which blocks key inflammatory signals (Gukovsky *et al*, 2003). Curcumin is an effective antioxidant and scavenges superoxide radicals, hydrogen peroxide and nitric oxide

form activated macrophages (Joe & Lokesh, 1994). It inhibits the inducible nitric oxide synthase activity macrophages (Brouet & Oshima, 1995). Human keratinocytes are protected from xanthine-xanthine oxidase injury by virtue of antioxidant property of curcumin (Bonte *et al*, 1997). Oral administration of 30 mg/kg body weight of curcumin in rats for 10 days reduces the iron induced hepatic damage by lowering lipid peroxidation (Reddy & Lokesh, 1996). Protection from radiation by dietary curcumin administered to mice is also attributed to antioxidant activity of curcumin. Curcumin protects renal cells and neural glial cells from oxidative stress. (Cohly *et al*, 1998) Interestingly curcumin not only shows antioxidative and free radical scavenging activities, but also enhances the activity of other antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase (Reddy & Lokesh, 1996). Lipid peroxidation is lower in liver, kidney, spleen and brain microsomes from retinol deficient rats that are fed with 0.1% dietary curcumin for three weeks (Kaul & Krishnakantha, 1997). Another mechanism by which curcumin protects against oxidative stress in endothelial cells is by the induction of heme oxygenase (Motterlini *et al*, 2000).

Cytochrome P450 species (CYP) are phase-I enzymes involved in activation of carcinogens whose inhibition adds a degree of cellular protection against cancer. It was previously published that curcumin inhibits alkylation reaction of ethoxyresorufin, methoxyresorufin and pentoxyresorufin catalyzed by CYP 1A1, 1A2 and 2B1 in rat liver (Thapliyal & Maru, 2001). Similarly, aflatoxin-DNA adducts formation, catalyzed by the CYP system, is inhibited by curcumin (Firozi *et al*, 1996). In DMBA-treated MCF7 cells, CYP activity is dramatically reduced by curcumin, which binds directly to the aryl hydrocarbon receptor (AhR) and thus prevents binding of this transcription factor to the

xenobiotic response element (XRE) present on the CYP gene promoter (Ciolino *et al*, 1998). Interestingly, Rinaldi *et al*. (2002) illustrated that curcumin activates the AhR while inhibiting carcinogen activation induced by CYP 1A1 in both oral SCC cells and intact oral mucosa. These authors suggest that the use of curcumin as an oral cavity chemopreventive agent could have a clinical impact via its ability to inhibit carcinogen bioactivation.

Evidence has also been presented that curcumin induces the activity of phase -II drug metabolizing enzymes in mice, particularly glutathione-S-transferase and quinone reductase in liver and kidney (Iqbal *et al*, 2003). However, this effect seems to be concentration-dependent and, if curcumin activates glutathione-S-transferase at low doses, high concentrations inhibit glutathione-S-transferase activity in rat (Piper *et al*, 1998). In the same way, Van Iersel *et al*. (1996) documented that curcumin is a strong glutathione-S-transferase inhibitor in human melanoma cells in which GSTP1-1 is the major isoform. Duvoix *et al*, confirms those results by providing evidence that curcumin significantly reduces GSTP1-1 expression in K562 and Jurkat leukemia cells by inhibiting NF-κB and AP-1 signaling pathways. In parallel, curcumin inhibits glutathione-S-transferase activity as well as CYP 1A1/1A2 in cells treated with different agents such as phenobarbital, β-naphthoflavone and pyrasol (Duvoix *et al*, 2003). Whereas in rat, curcumin allows the restoration of normal levels of GSTP in hepatic cells (Oetari *et al*, 1996).

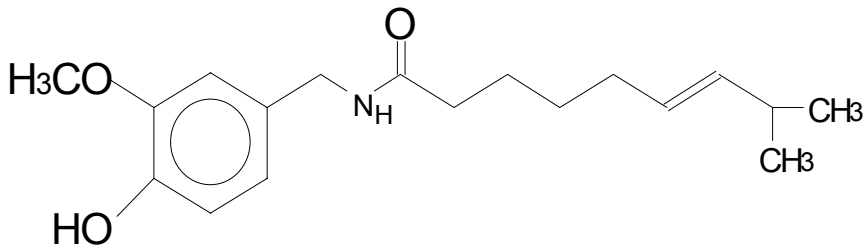
In diabetic rats, dietary curcumin improves their metabolic status (Babu & Srinivasan, 1995). Diabetic rats maintained on a 0.5% curcumin diet for 8 weeks excreted comparatively lower amounts of albumin, urea, creatinine, inorganic phosphorous, sodium and potassium. On the other hand, glucose excretion or the

fasting sugar level was unaffected by dietary curcumin and also the bodyweights were not improved to any significant extent. Diabetic rats fed a curcumin diet had a lower relative liver weight at the end of the study, compared to other diabetic groups. Diabetic rats fed a curcumin diet also showed lowered lipid peroxidation in plasma and urine (Babu & Srinivasan, 1998). Curcumin is also highly effective in inhibiting type-I HIV, long terminal repeat directed gene expression and viral replication (Jiang *et al*, 1996). Curcumin inhibited p24 antigen production in cells either acutely or chronically infected with HIV-I (Li *et al*, 1993).

Several studies reveal that curcumin glucuronoside, dihydroglucuronoside, tetrahydro glucuronoside and tetrahydro-curcumin are the major metabolites of curcumin *in vivo* (Pan *et al*, 1999). It is proved that, curcumin associates with serum albumin through hydrophobic interactions (Reddy *et al*, 1999) thereby, be transported to appropriate target cells, where it elicits its pharmacological actions.

A recent review (Joe *et al*, 2004) rightly termed curcumin as 'wonder compound' having plethora of beneficial effects and certainly qualifies for serious consideration as a pharmaceutical / nutraceutical / phytoceutical agent.

2.2 Capsaicin



Hot chili peppers that belong to the plant genus *Capsicum* (family *Solanacea*) are among the most heavily consumed spices throughout the world (Govindarajan & Satyanarayana, 1991). The primary pungent principle in *Capsicum* fruits is identified as capsaicin (8-methyl-N-vanillyl-6-nonanamide) (Monsereenusorn *et al*, 1982). The capsaicin content of green and red peppers ranges from 0.1 to 1%. Capsaicin evokes numerous biological effects and thus has been the target of extensive investigations since its initial identification in 1919.

One of the most recognized physiological properties of capsaicin is its selective effects on the peripheral part of the sensory nervous system, particularly on the primary afferent neurons of C-fibre type (Dray, 1992). The compound is known to deplete the neurotransmitter of painful impulses known as substance P from the sensory nerve terminals, which provides a rationale for its use as a versatile experimental tool for studying pain mechanisms and also for pharmacotherapy to treat some peripheral painful states, such as rheumatoid arthritis, post-herpetic neuralgia, post-mastectomy pain syndrome and diabetic neuropathy (Lotz, 1994). With regard to peripheral neurogenic influence, capsaicin is capable of both blocking neurogenic inflammation and pain. Its stimulating and desensitizing effects show graded responses depending upon the dosage used and time elapsed after treatment. Recently, capsaicin has received considerable attention as a pain reliever. In two trials with 70 and 21 patients with osteoarthritis and rheumatoid arthritis, topical application of 0.025% or 0.07% capsaicin was effective and safe alternative to analgesics employed in systemic medications, which are often associated with potential side effects (Deal, 1991; McCarthy & McCarthy, 1991). Capsaicin has also been suggested for the initial management of neuralgia consequent to herpes infection (Bernstein,

1989). Clinical trials revealed that topical cream containing 0.025% capsaicin significantly ameliorated the pain in patients with arthritis (Cordell & Araujo, 1993).

Extensive research in the last few years has shown that the pathway that activates nuclear transcription factor kappa-B transcription factor can be interrupted by capsaicin (Aggarwal & Shishodia, 2004). Significant number of reports indicates that regular consumption of capsaicin can reduce the risk of acquiring specific cancers (Dorai & Aggarwal, 2004).

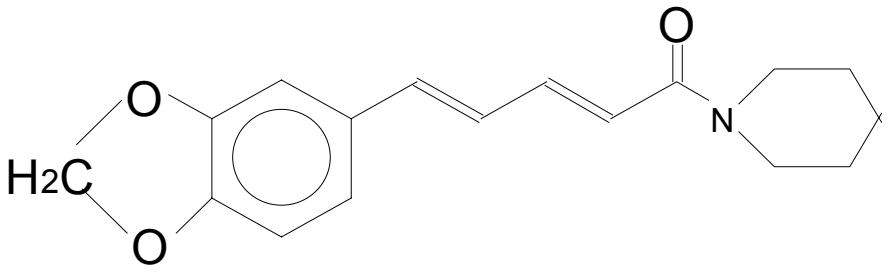
Another field of intensive studies in recent times is diabetic neuropathy. Capsaicin has been shown to be useful in diabetic neuropathy (The Capsaicin Study Group, 1992). In an eight week, double blind, placebo-controlled study with parallel randomized treatment conducted by 12 independent investigators involving 219 patients, topical application of 0.075% capsaicin cream was effective in pain management. A recent experimental data shows that capsaicin ineffective in relieving pain associated with HIV-related peripheral neuropathy (Paice, 2000). Mason et al. have attempted to determine the efficacy and safety of topically applied capsaicin for chronic pain from neuropathic or musculoskeletal disorders. They have revealed that although topically applied capsaicin has moderate to poor efficacy in the treatment of chronic musculoskeletal or neuropathic pain. How ever it may be useful as an adjunct or sole therapy for a small number of patients who are unresponsive to, or intolerant of, other treatments (Mason *et al*, 2004).

Capsaicin has achieved widespread use in clinical research because it induces cough in a dose-dependent and reproducible manner. A review of the 20-year clinical experience has failed to uncover a single serious adverse event associated with

capsaicin cough challenge testing in humans safety of capsaicin cough challenge testing (Dicpinigaitis *et al*, 2005).

Capsaicin, a dietary hypolipidemic spice principle of red pepper was found to be beneficial in protecting the structural integrity and fluidity of erythrocytes under conditions of hypercholesterolemia and hypertryglyceridemia (Kempaiah & Srinivasan, 2002; Kempaiah & Srinivasan, 2005). Capsaicin offer this beneficial effect by correcting the altered cholesterol to phosphorus ratio in the erythrocytes in both hypercholesterolemic and hypertryglyceridemic condition (Kempaiah & Srinivasan, 2002; Kempaiah & Srinivasan 2006). Spice principle capsaicin is evidenced to inhibit oxidation of human low-density lipoprotein *in vitro* (Naidu & Thippeswamy, 2002). Dietary capsaicin has been observed to have a protective influence on induced oxidation of low-density lipoprotein in rats (Kempaiah *et al*, 2005).

2.3 Piperine



Piperine (1-Piperoyl piperidine) is a pure, non-toxic pungent alkaloid constituent of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). Many health beneficial physiological effects are attributed for this compound. The most far-reaching attribute of piperine has been its inhibitory influence on enzymatic drug biotransforming

reactions in liver, which results in enhancing the bioavailability of a number of therapeutic drugs as well as phytochemicals (Srinivasan, 2006). Piperine has been reported as bioavailability modulator by several investigators. Shobha *et al*. (1998) revealed that, piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects. Badmaev *et al*. (2000) have investigated that supplementation of 120 mg coenzyme Q_{10} with piperine for 21 days produced a significant, approximately 30% greater plasma concentration was observed during supplementation with coenzyme Q_{10} plus placebo. It is postulated that the bioenhancing mechanism of piperine to increase plasma levels of supplemental coenzyme Q_{10} is nonspecific and possibly based on its description in the literature as a thermo nutrient. Hiwale *et al*. (2002) found that co-administration of piperine enhanced bioavailability of beta lactam antibiotics, amoxycillin trihydrate and cefotaxime sodium significantly in rats. The increased bioavailability is attributed to the effect of piperine on microsomal metabolizing enzymes or enzymes system.

Khajuria *et al*. (1998) have pointed out that piperine may act as an apolar molecule and form apolar complex with drugs and solutes. It may modulate membrane dynamics due to its easy partitioning thus helping in efficient permeability across the barriers. Khajuria *et al.* (2002) also pointed out that piperine may be inducing alterations in membrane dynamics and permeation characteristics, along with induction in the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier.

Piperine treatment has also been evidenced to lower lipid peroxidation and beneficially influence cellular antioxidant status in a number of experimental situations of oxidative stress. The studies by Khajuria *et al*. (1998a) indicate a protective role of piperine against the oxidative alterations by carcinogens. It is also suggested that piperine modulates the oxidative changes by inhibiting lipid peroxidation and mediating enhanced synthesis or transport of GSH thereby replenishing thiol redox.

Lin *et al*. (1999) have revealed that black pepper extract was found to possess growth-stimulatory activity towards cultured melanocytes. Its aqueous extract at 0.1 mg/ml was observed to cause nearly 300% stimulation of the growth of a cultured mouse melanocyte line, melan-a, in 8 days. Piperine also significantly stimulated melan-a cell growth. Piperine induced morphological alterations in melan-a cells, with more and longer dendrites observed. Sunila *et al.* (2004) have studied alcoholic extract of the fruits of the *Piper longum* and piperine was studied for their immunomodulatory and antitumor activity. Selvendiran *et al.* (2004) have found that administration of piperine significantly decreased the levels of lipid peroxidation, protein carbonyls, nucleic acid content and polyamine synthesis that were found to be increased in lung cancer bearing Swiss albino mice. It is inferred that piperine could effectively inhibit benzpyrene-induced lung carcinogenesis in albino mice by offering protection from protein damage and also by suppressing cell proliferation. Selvendiran *et al*. (2005) have revealed that lung cancer bearing mice showed a significant decrease in the activities of mitochondrial enzymes - isocitrate dehydrogenase α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase and significantly increased NADPH-Cytochrome reductase, cytochrome P_{450} and cytochrome b_5. The activities of glutathione-metabolizing enzymes glutathione

peroxidase, glutathione reductase and glucose-6-phosphodehydrogenase were significantly lowered in lung cancer bearing mice when compared with control mice. Piperine supplementation to tumour-induced animals significantly lowered the phase-I enzymes (NADPH-C reductase, cyt-P_{450} and cyt-b_5)) and there was a rise in glutathione-metabolizing enzymes (GPx, GR and G6PDH), which indicated an antitumour and anti-cancer effect.

Malini *et al*. (1999) have reported that histological studies with piperine showed that a 5 mg dose caused partial degeneration of germ cell types, whereas at a 10 mg dose, it caused severe damage to the seminiferous tubule, decrease in seminiferous tubular and Leydig cell nuclear diameter and desquamation of spermatocytes and spermatids. Correlated to the structural changes, a fall in caput and cauda epididymal sperm concentrations was also evident. A 10 mg dose of piperine also caused a marked increase in serum gonadotropins and a decrease in intra-testicular testosterone concentration, despite normal serum testosterone titres. A report (Daware *et al*, 2002) points out that piperine interferes with several crucial reproductive events in a mammalian model. Piperine (10 and 20 mg/kg b.w.) increased the period of the diestrous phase which seemed to result in decreased mating performance and fertility. Post-partum litter growth was not affected by the piperine treatment. Sperm shape abnormalities were not induced by piperine at doses up to 75 mg/kg b.w. Considerable anti-implantation activity was recorded after five days post-mating oral treatment with piperine. The sex ratio and post-implantation loss were unaffected after treatment with piperine. Intrauterine injection of piperine caused the total absence of implants in either of the uterine horns (16.7%) or one of the horns (33%) of treated females. No histopathological changes were detected in the ovary and the uterus at the cellular

level. D'cruz and Mathur (2005) have reported that piperine caused a decrease in the activity of antioxidant enzymes and sialic acid levels in the epididymis and thereby increased reactive oxygen species levels that could damage the epididymal environment and sperm function.

Another aspect of piperine which has been studied extensively is its inhibitory effect on mixed function oxygenase system. Singh *et al*. (1986) have reported that piperine inhibits the activities of rat hepatic monooxygenases and UDP-glucuronyl transferase. The studies also demonstrate that piperine modifies the rate of glucuronidation by lowering the endogenous UDP-glucuronic acid content and also by inhibiting the transferase activity. Another report (Sambaiah & Srinivasan, 1989) reveals that piperine stimulates liver microsomal cytochrome P_{450}-dependent aryl hydroxylase. Dalvi and Dalvi (1991) reported that an intragastric dose of 100 mg/kg of piperine to adult, male Sprague-Dawley rats caused an increase in hepatic microsomal cytochrome P-450 and cytochrome b_5, NADPH-cytochrome-C reductase, benzphetamine N-demethylase, aminopyrine N-demethylase and aniline hydroxylase 24 h following treatment. On the other hand, a 10 mg/kg dose given intra-peritoneally exhibited no effect on the activities of the aforementioned parameters of the hepatic drug-metabolizing enzyme system. However, when the intragastric and intra-peritoneal doses were increased to 800 mg/kg and 100 mg/kg, respectively, the black pepper alkaloid produced a significant decrease in the levels of cytochrome P_{450}, benzphetamine N-demethylase, aminopyrine N-demethylase and aniline hydroxylase 24 h after treatment. None of the treatments significantly elevated the activities of serum sorbitol dehydrogenase (SDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and isocitrate dehydrogenase (ICD), suggesting that piperine

is not a hepatotoxic agent. Selvendirn *et al*. (2005) examined the protective role of piperine during experimental lung carcinogenesis with reference to its effect on DNA damage and detoxification enzyme system. The activities of detoxifying enzymes such as glutathione transferase (GST), quinone reductase (QR) and UDP-glucuronosyl transferase (UDP-GT) were found to be decreased while the hydrogen peroxide level was increased in the lung cancer bearing animals. Supplementation of piperine (50 mg/kg b.w.) enhanced the detoxification enzymes and reduced DNA damage as determined by single cell electrophoresis. Furthermore, the DNA-Protein cross links which was found to be high in lung cancer bearing animals was also modulated upon supplementation with piperine. These results explain the understanding of unique association between anti-peroxidative effect of piperine and ultimately the capability of piperine to prevent cancer.

Like other spice principles, piperine is also known for its digestive stimulant activity. Piperine was examined for its possible influence on digestive enzymes of intestinal mucosa in experimental rats. It enhanced intestinal lipase activity and also the disaccharidases - sucrase and maltase (Platel & Srinivasan, 1996). Another study (Platel & Srinivasan, 2000) targeted at possible influence of piperine on digestive enzymes of pancreas in experimental rats. Piperine prominently enhanced pancreatic lipase and trypsin activity and also stimulated pancreatic amylase and chymotrypsin activity. This stimulatory influence of piperine on the pancreatic digestive enzymes was however not observed when their intake was restricted to a single oral dose. This is further evidenced by an *in vitro* experiment (Ramakrishna Rao *et al*, 2003) in which piperine effect was examined on the activities of digestive enzymes of rat pancreas and small intestine by including them in the reaction mixture. It enhanced the activity

of pancreatic lipase and amylase. It is inferred that the positive influences of piperine on these enzymes of digestive process could be an additional feature of the parent spice, black pepper that is generally well recognized to stimulate digestion.

Few investigators have reported the metabolism, tissue distribution and excretion of piperine by different routes of administration. A study (Bhat & Chandrasekhara, 1986) indicates that about 97% of the dose was absorbed when it was administered by orally and intra-peritoneally. Three per cent of the administered dose was excreted as piperine in the feces. Piperine was not detectable in urine. When everted sacs of rat intestines were incubated with 200-1000 mg of piperine, about 47-64% of the added piperine disappeared from the mucosal side. Only piperine was present in the serosal fluid and also the intestinal tissue, indicating that piperine did not undergo any metabolic change during absorption. It is reported that (Bhat & Chandrasekhara, 1987). After oral administration of piperine, four metabolites of piperine, viz. piperonylic acid, piperonyl alcohol, piperonal and vanillic acid were identified in the free form in 0-96 h urine whereas only piperic acid was detected in 0-6 h bile. Bajad *et al.* (2003) reported a new major urinary metabolite in rat urine and plasma using HPLC. The metabolite has a unique structure compared to the previously reported metabolites in that it retains methylenedioxy ring and conjugated double bonds while the piperidine ring is modified to form propionic acid group. A pharmacokinetic study (Sunkara *et al*, 2001) states that, after bolus intravenous administration of piperine at a dose of 10 mg/kg, the serum concentration - time curve fitted the two-compartment open model. The tissue distribution pattern of piperine in rats also supports the two-compartment open model.

3. Bioavailability of bioactive compounds

Bioavailability is defined as a measure of the rate and amount of the compound of interest which reaches the systemic circulation unchanged following its administration. Various factors can alter the bioavailability, among which most critical is the chemical structure of the compound which usually determines the major properties that could influence significantly its dissolution and bioavailability. It is not the lipid solubility of the compound, but rather its water / lipid partition co-efficient that is the limiting factor in the absorption process. Under optimum conditions, therefore, the compound should have only sufficient water solubility to dissolve in the aqueous phase and lipid solubility high enough to prevent entrainment in the water layer. This will facilitate its penetration through lipoidal barrier of gastrointestinal tract and in to the blood stream. Although bioavailability can play a key role in explaining intra- and inter-subject variability in compound blood concentrations, it is by no means the sole factor. Compound elimination process, including metabolism and excretion also are major contributing factors.

Another important property of the compound is its permeability related characteristics. A compound should have good capability to cross the liquid barriers of the cell membranes at the absorption site to enter the systemic circulation. Compound binding to serum albumin and to enzymes of Mixed Function Oxygenase System can also reduce bioavailability (through sequestration in the plasma or rapid compound modification) and increase the risk of drug-drug interactions (by releasing the albumin bound drugs or affecting the normal oxidative modification of other compounds). However another consequence of compound modification through oxidative enzymes

is the creation of bioactive compounds that can form protein - drug adducts and results in organ toxicity, particularly in the liver (Evans *et al*, 2004).

The extent and the rate of absorption of the compound play an important role in pharmacokinetics. For example, a fraction of the dose may be metabolized during the early passage through the gastrointestinal tract or through the liver after an oral dose, or part of the dose may not reach the blood due to malabsorption. The consequence is an incomplete absorption of the compound into the systemic circulation and incomplete availability of the compound may produce ineffectiveness of the treatment.

Absorption is a complex process which cannot be monitored experimentally in a simple way, and consequently it is not easy to get the extent of drug absorption by direct observation. Anyway pharmacokinetic models allow estimating this parameter with a simple experimental design. Looking at the definition of clearance, it is clear that having the plasma Area under the Curve (AUC) after any route of drug administration and knowing Clearance, it is always possible to compute the drug amount which enters into the systemic circulation in a particular subject after any route of administration. Then, an easy way to perform a bioavailability experiment is to treat a subject both, intravenously to get clearance, and orally (or by any other test route) to get the AUC.

Pharmacokinetic profile of a compound includes the absorption, the distribution, the biotrasformations and the elimination in man and animals. Absorption and distribution indicate the passage of the drug molecules from the administration site to the blood and the passage of drug molecules from blood to tissues respectively. Drug elimination may occur through biotrasformation and by the passage of molecules from the blood to the outside of the body through urine, bile (and subsequently feces) or other routes.

Measuring the amounts or the concentrations of drugs in blood, urines or other fluids or tissues at different times after the administration, much information can be obtained on drug absorption and on the passage of drug molecules between blood and tissues and finally on the drug elimination. Knowledge of the pharmacokinetics and of the effects (pharmacodynamics) of drugs in man is necessary for an optimal use of drugs in therapy (choice of the best route of administration, choice of the best dose regimen, dose individualization).

Many orally administered phyto-nutrients, drugs must overcome several barriers before reaching their target site. The first major obstacle to cross is the intestinal epithelium. Although lipophilic compounds may readily diffuse apical plasma membrane, their subsequent passage across the basolateral membrane and into the blood is by no means guaranteed. Efflux proteins located at apical membrane, which include P-glycoprotein (a multi drug resistance protein) may drive compounds from inside the cell back into the intestinal lumen, preventing their absorption in to blood.

The efficacy of many drugs depends critically on their ability to cross cellular barriers to reach their target. Lipophilic drugs may cross these barriers in the absence of specialized transport systems, since these compounds diffuse freely across the plasma membrane. Hydrophilic and charger compounds on the other hand often require specific transport mechanisms to facilitate cellular uptake and/or transcellular transport. However, the extent to which a drug accumulates within a tissue is frequently limited not so much but its ability to enter cells but by its tendency to leave. This may arise from the active efflux mechanisms present I the plasma membrane. These efflux mechanisms play a critical role in limiting the absorption and accumulation

of potentially toxic substances and can effectively confer resistance to diverse range of compounds in tumor cells.

3.1 Intestinal absorption

The small intestine represents the principal site of absorption for any ingested compound, whether dietary, therapeutic, or toxic. Oral administration is the most popular route for drug administration since dosing is convenient and non-invasive and many drugs are well absorbed by the gastrointestinal tract. As well as degrading and absorbing nutrients and solutes from the intestinal lumen, intestinal enterocytes from a selective barrier to drugs and xenobiotics. This barrier function depends largely upon specific membrane transport systems and intracellular metabolizing enzymes. The extent to which a compound is absorbed by the intestinal epithelium is therefore a critical factor in determining its overall bioavailability.

There are two principal routes by which compounds may cross the intestinal epithelium: paracellular or transcellular. A number of small, hydrophilic, ionized drugs are absorbed via the paracellular pathways. However, absorption via this route is generally less since intercellular tight junctions restrict free transepithelial movement between epithelial cells. Transcellular absorption from lumen to blood requires uptake across the apical membrane, followed by transport across the cytosol, then exit across the basolateral membrane and into blood. The transcellular absorption of hydrophilic drugs may be facilitated via specific carrier mediated pathways by means of utilizing the same route of absorption followed by nutrients and micronutrients. Many orally administered drugs are lipophilic and undergo passive transcellular absorption (Hunter & Hirst, 1997). Drugs that cross the apical membrane may be substrates for apical efflux transporters, which extrude compounds back into the lumen (Evers *et al*, 1998).

These apical efflux transporters are principally ABC proteins such as Pgp and MRP2, and are ideally situated to act as the first line of defense by limiting the absorption of potentially toxic foreign compounds. Compounds that are already present in the blood may undergo active blood-to-lumen secretion facilitated by these transporters. As well as efflux pumps, the transcellular route of absorption exposes drugs to intracellular metabolic systems; small intestinal enterocytes provide the first site for cytochrome P450-mediated metabolism of orally ingested drugs and xenobiotics (Watkins, 1992). The CYP system (phase I metabolism), plus other intracellular metabolic systems, such as phase II conjugating enzymes, may yield metabolites that are themselves substrates for efflux pumps, thus providing additional possibilities for interactions.

Because of the economical and ethical limitations of *in vivo* studies, several *in vitro* models and procedures have been introduced to assess the major factors involved in the absorption process and help predict how well the drug will be absorbed and whether the absorption is a dissolution limited or permeability limited process. These *in vitro* procedures may be of extreme value, especially during the screening of new drug candidates. For many drugs, pharmacologic response and therapeutic effectiveness can be related directly to the observed levels.

In recent past there are number of reports demonstrating the cutting edge technology applications for the enhancement of drug delivery. Bianco (2004) defines the use of functionalized carbon nanotubes (f-CNTs) as new carrier systems for the delivery of therapeutic molecules, antigens and genes. Shimpi *et al*. (2005) have discussed the usefulness of cyclodextrins in the different routes of drug administration. Many studies have shown that cyclodextrins are useful additives in the different routes of drug administration because of increased aqueous solubility, stability, bioavailability

and reduced drug irritation. Pathak *et al*. (2005) have explained the importance of various supercritical fluid technologies for enhancement of drug bioavailability. Sapra *et al*. (2005) have pointed out the application of selective targeting of ligand targeted liposomes containing anticancer drugs or therapeutic genes to cell surface receptors expressed on cancer cells is a recognized strategy for improving the therapeutic effectiveness of conventional chemotherapeutic or gene therapeutics. Ligand targeted liposomes have shown considerable promise in preclinical xenograft models and are poised for clinical development. Micelles are currently successfully used for the solubilization of various poorly soluble phyto and pharmaceuticals and demonstrate a series of attractive properties as drug carriers (Torchilin, 2005).

Liposomes or micelles are spherical particles that encapsulate a fraction of the solvent, in which they freely diffuse (float) into their interior. They can have one, several or multiple concentric membranes. Liposomes are constructed of polar lipids which are characterized by having a lipophilic and hydrophilic group on the same molecules (Bangham & Horne, 1964; Lasic, 1993). Upon interaction with water, polar lipids self-assemble and form self-organized colloidal particles. Simple examples are detergents, components form micelles, while polar lipids with bulkier hydrophobic parts cannot associate into micelles with high curvature radii but form bilayers which can self-close into liposomes or lipid vesicles. Consequently, water soluble compounds are entrapped in the water compartment and lipid soluble compounds aggregate in the lipid section. Uniquely, liposomes can encapsulate both hydrophilic and lipophilic materials. Liposomes resemble the lipid membrane part of cells. Numerous biological processes in living organisms depend on the action of small unilamellar liposomes. Typical compositions include lecithin (phosphatidyl cholines), and cephalins (phosphatidyl-

ethanolamines) often containing negatively charged lipids, such as phosphatidyl serine and phosphatidyl inositol. In addition to ceramides, such as sphingomyelin; sterols (cholesterol, ergosterol, sitosterol, etc.) are also included. Recently, the use of synthetic, most often nonionic, lipids are starting to increase (Vanlerberghe, 1996). With respect to the number of lamellae and size of liposomes, small unilamellar vesicles have a size <0.1 mm, large bilamellar vesicles have a size range of 0.1–1 mm, and large multilamellar vesicles can be up to 500 mm in diameter and can contain hundreds of concentric bilayers (Helfrich, 1984). Because of their structure and composition, liposomes are used extensively in basic research as a model for biological membranes. Due to their biocompatibility, biodegradability and absence of toxicity, in applications ranging from drug and gene delivery to diagnostics, and the food industry (Dufour *et al*, Year).

3.2 Transport of Lipophilic bioactive compounds in blood circulation

Serum albumin has a well-known affinity for variety of saturated and unsaturated fatty acids of varying chain lengths (Brown & Shockley, 1982). It is recognized as the principal transport protein for fatty acids and other lipids that would otherwise be insoluble in the circulating plasma. The crystal structure of human serum albumin (HSA) complexed with fatty acids demonstrated that fatty acid binding is accompanied by a significant conformational change in the protein (Curry *et al*, 1998). The dramatic conformational change has both global and local aspects. Globally, fatty acid binding induces relative rotations of the three domains of the protein; these are almost rigid-body movements since there is only very modest distortion of the individual domains upon binding. While the global changes are quite dramatic, the local effects of fatty acid binding on the protein are more subtle as there is no significant main chain

movement and the principal local adjustments are limited to rotations of side chains to make way for the incoming ligand. The physiological significance of conformational change induced by fatty acid binding, if any, is not known. Conceivably, it may allow HSA receptors to discriminate between loaded and empty HSA molecules in order to facilitate the efficient delivery of fatty acids to cells. Evidence suggests the existence of number of HSA receptors on cell surfaces, but their precise role in fatty acid transport is not universally accepted and the issue remains contentious.

It can bind to a wide variety of endogenous and exogenous drugs means that protein can have major effect on pharmacokinetics. Many of interactions are through hydrophobic forces and some are due to the conformational flexibility of the albumin to accommodate ligands. Ligands bind to HSA both strongly and weakly and most of them are reversible. If the binding constant is of the order of $10^3 M^{-1}$, nearly 50% of drug in the serum compartment will be bound to HSA. Up to a point, this is an advantageous property as albumin binding increases the drug bioavailability by decreasing the clearance rate. However, when the binding constant is $> 10^8 M^{-1}$, most of the drug in the serum is albumin bound and the in-vivo efficacy can be significantly reduced. The other disadvantage of this pharmacokinetic profile is that a highly bound drug is susceptible to severe drug-drug interactions. When the amount of free drug is very small compared to the amount that is protein bound, any situation that disrupts albumin binding can significantly increase the concentration of free drug.

Human serum albumin is a heart shaped monomeric non-glycoprotein of 585 amino acids with molecular weight of 66.5 kDa. The protein is 67% α-helical and entirely lacking in β-sheet. As predicted by sequence analysis, HSA is organized in to three

homologues domains, I (residues 1-195), II (196-383) and III (384-585), which have similar 3D structures (Meloum *et al*, 1975) Each domain can be divided into two sub domains A and B, which are formed from six and four α-helices, respectively. It contains 17 disulfide bridges, a free thiol (Cys-34) and a single tryptophan (Trp-214). HSA contains high content of charged amino acids such as aspartic acid, glutamic acid, lysine and arginine (Ho & Carter, 1992; Carter & Ho, 1994)

HSA has 76% homology with bovine serum albumin and rat serum albumin (Peters, 1985). All the 17 disulfides are strictly conserved in human, bovine, rat, ovine, salmon, lamprey and horse serum albumin giving a common topology to the molecules. Despite the great homology in structure there are marked differences in the binding and other functional properties among albumen (Ho *et al*, 1993). Albumin stability is mainly attributed to a large number of disulfide bridges. The 17 disulfides are exclusively intradomain and conserved across species, contributes towards HSA`s impressive thermo stability. Despite very high stability, HSA is a flexible protein with the 3D structure susceptible to environmental factors such as pH, ionic strength, etc. (Wallevik, 1973).

The ligand binding sites are mainly classified as 3 groups based on the ligand. Site 1 and 2 are located on sub domain IIA and IIIA which are specific for small organic compounds (Peters, 1985a). Even though the amino acid distribution and the charge density are quite similar but exhibit remarkable specificity for ligands. The major endogenous ligand, which binds to IIA, is bilirubin with K_a of 1.4×10^8 M^{-1}. This high affinity prevents the entry of bilirubin to the nervous system (Jacobsen & Brodersen, 1983). Site 3 & 4 are principal carriers of fatty acid in albumin. Under normal physiological conditions HSA carries one or two fatty acids of same or different length.

One of the probable binding sites is IIIA as shown by the competitive binding studies with marker ligands (Rechieri *et al*, 1993). Site 5 and 6 are called metal ion binding sites. Here the major serum metals such as mercury, copper, zinc and aluminium will bind reversibly. Among the metals, calcium and zinc have low affinity for albumin. The site 6 is which is part of sub domain IB is responsible for binding of copper and nickel (Dixon & Sarkar, 1974).

4. Mixed Function oxygenase system

MFOS is a complex of enzymes present in the endoplasmic reticulum of mammalian cells which involve in the hydroxylation of endogenous steroids besides its involvement in the primary metabolism of foreign compounds such as phytonutrients, xenobiotics, drugs etc. The most important site of metabolism mediated by MFOS is the liver. In humans, at least 57 different CYP isoforms have been identified, with broad and overlapping substrate specificity. Ongoing research has documented the nearly ubiquitous involvement of CYPs in the metabolism of small organic molecules.

Metabolism of foreign molecules occurs in two phases. Hydroxylation is the major reaction involved in phase-I. This reaction is catalyzed by a group of enzymes referred to as monooxygenases or Cytochrome P-450 species. Other types of reactions occurring in phase-I are de-alkylation, reduction and hydrolysis. In phase-II, the hydroxylated or other compounds produced in phase-I are converted by specific enzymes to various polar metabolites by conjugation. Conjugation of compounds can occur either with glucuronic acid, sulfate, acetate, glutathione or certain amino acids. In fact, it is currently estimated that the Cytochrome P-450 enzymes are responsible for ~90% of the phase-I metabolism of drugs.

The overall purpose of the two phases of metabolism of foreign compounds is to increase their polarity and thus facilitate their excretion from the body. The process is known as detoxification. Hydrophobic foreign compounds would persist in adipose tissue almost indefinitely if they are not converted to more polar forms.

There are two important outcomes of the metabolism of foreign compounds by MFOS. First, is often the case, the compound is itself a substrate for at least one of the enzymes, resulting in rapid oxidation of the molecule. If the oxidized product is inactive, this leads to rapid clearance and low bioavailability of the active compound. It can sometimes be the case that the oxidized product retains its activity, or even that the administered compound is inactive until acted upon by these enzymes. Such process is called bioactivation. A second major outcome is that MFOS activation or inhibition. This can result in potentially undesirable interactions of foreign compounds by enhancing or preventing the metabolism and altering the pharmacokinetic profile of second compound that is acted upon by the particular enzyme. While MFOS inhibition is generally undesirable, this can be exploited to boost the pharmacokinetic profile of co-administered compound.

The involvement of the liver microsomal MFOS in the metabolism of drugs, pesticides and other foreign compounds is well documented. It has been concluded that the induction of mixed function oxidation of xenobiotics is an adaptation in animals for the detoxification of dietary lipophilic toxicants of low, but appreciable toxicity (Hodgson, 1974). This stimulation of drugs, xenobiotics etc on their own metabolism often allows the organism to detoxify them more rapidly. Induction is frequently accompanied by increase in liver size, the extent of which depends upon the dose and

duration of exposure. This increase in liver size is mainly because of the proliferation of smooth endoplasmic reticulum of liver cells, which houses the components of MFOS i.e., the enzyme systems for detoxification and probably also the excretion system for the detoxified hydrophilic metabolites. Proliferation of endoplasmic reticulum is associated with increased rate of microsomal protein synthesis.

Inducers of microsomal enzymes stimulate the metabolism or synthesis of several normal body constituents such as steroid hormones, pyridine nucleotides, cytochromes and bilirubin. Steroids are the normal body substrates of drug metabolizing enzyme system in liver microsomes. Treatment of rats with Phenobarbital enhances hydroxylation of various steroid hormones such as androgens, estrogens, glucocorticoids and progestrational steroids by liver microsomes. Such an effect would enhance the metabolism of steroids to more polar metabolites and hence decreases the hormonal action of steroids such as estradiol, estrone and progesterone. Inducers of liver microsomal enzymes have been found to enhance the hydroxylation of steroids in human beings also.

Since the first three-dimensional structure of the bacterial CYP101 was elucidated by Poulos *et al* (1985) several other structures have been resolved including microsomal ones, CYP2B4 (Scott *et al*, 2003; Werck-Reichhart & Feyereisen, 2000). The active center is the iron-protoporphyrin IX with an axial thiolate of a cysteine residue as fifth iron ligand. In the absence of a substrate at the beginning of the cycle CYP is in the hexa-coordinated low-spin ferric form with water being the sixth ligand. The overall reaction of substrate hydroxylation of the CYP monooxygenase function is the insertion of one atom of the oxygen molecule into an substrate RH, the second

atom of oxygen being reduced to water while consuming two reducing equivalents under formation of ROH.

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$$

The electrons are delivered by flavoproteins or ferredoxin like proteins and NAD(P)H in a complex electron transfer chain. The most generally accepted mechanism for substrate hydoxylation by CYP includes the following steps although several details remain still unsolved (Auclair et *al*, 2002). Substrate binding to the hexa-coordinated low-spin ferric enzyme excludes water from the active site, which is causing a change to the 5-coordinate high-spin state. The decrease of polarity is accompanied with a positive shift of the redox potential by about 130 mV that makes the first electron transfer step thermodynamically favourable. The transfer of one electron from a redox partner reduces the ferric iron to the ferrous enzyme. This can now bind molecular oxygen forming a ferrous-dioxygen (Fe^{2+}-O_2) complex. The second electron is transferred along with a proton gaining an ironhydroperoxo (Fe^{3+}–OOH) intermediate. The O - O bond is cleaved to release a water molecule and a highly active ironoxo ferryl intermediate. This intermediate abstracts one hydrogen atom from the substrate to yield a one-electron reduced ferryl species and a substrate radical or reacts in a concerted reaction with the substrate C - H bond without intermediate radical formation. Then, it follows immediately the enzyme-product complex formation and release of the product ROH to regenerate the initial low-spin state. The iron-oxo intermediate may however also induce the formation of protein radicals (Sch¨unemann *et al*, 2002)

In man there are around 30 CYP enzymes called CYP supra family which are responsible for drug metabolism and these belong to families 1–4. It has been

estimated, however, that 90% of drug oxidation can be attributed to six main enzymes: CYP 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 (Tanaka, 1998). The most significant CYP isoenzymes in terms of quantity are CYP3A4 and CYP2D6. CYP3A4 is found not only in the liver but also in the gut wall, where it may serve as a primary defense mechanism. The bulk of drugs acting on the CNS, with the exception of volatile anaesthetic agents, are metabolized by this enzyme.

Among the earliest discoveries of the effects of diet on enzyme activity were those made by Wattenberg (Wattenberg, 1971). By manipulating the diet of rats, he discovered that the addition of food supplements containing cruciferous vegetables, such as cabbage and Brussel sprouts, could increase the activity of both CYP1A1 and CYP1A2 by a factor of 70. A further example relates to induction of CYP2B1 by diallyl sulfide in garlic (Brady et al, 1991). In recent years, a number of alternative medicines including herbal remedies such as St John's Wort, which is used for mild depression, have gained increasing popularity. St John's Wort is known to interfere with the metabolism of a large number of medicines due to its ability to induce CYP3A4. Another important cause of morbidity due to enzyme inhibition is citrus fruit. The most important of these is grapefruit juice, which contains a number of potent CYP enzyme inhibitors. These include the plant alkaloids naringin, naringenin and bergamottin. In particular, CYP3A4 is inhibited, leading to altered drug disposition of a number of substances including the antihistamine terfenadine, which can result in fatal cardiac arrhythmia (Ameer & Weinttraub, 1997). Atmospheric pollution is also a cause of enzyme induction. Anaesthetists are continuously exposed to low-level pollution of the atmosphere by volatile agents. It has been shown that the elimination of a number of drugs in this group is increased by up to 29% (Duvaldestin *et al*, 1981).

5. Scope of Present Investigation

Heat processing of foods in addition to altering physical, chemical and nutritional nature of starches and proteins, also changes the bioavailability of proteins, carbohydrates, lipids and vitamins. Since, many health beneficial physiological effects of spices are attributable to their active principles there is a need to evaluate the availability of the spice active principles in their original form when spices are heat processed as in domestic cooking. Considerable extent of loss if any in the concentration of active principles would raise a question as to what extent the spices would retain their health beneficial effects after their conventional heat processing as in domestic food preparation. In this context, three spices – turmeric (*Curcuma longa*), red pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) were evaluated for the status of their active principles during domestic food processing with variables such as presence of pulses and acidulants.

It was observed during this investigation that when spices turmeric, red pepper and black pepper are heat processed as in domestic cooking, a considerable extent of loss in the concentration of respective spice active principles would occur. Hence, efforts were made to structural characterize the altered / degraded compounds arising from these spice principles.

In view of the multiple health beneficial physiological effects which curcumin, capsaicin and piperine are known to elicit, there is a need to evaluate the bioavailability of these spice active principles. Curcumin is understood to possess low systemic bioavailability. This pharmacokinetic feature of curcumin, which has been observed in several species, is attributed to a generally poor absorption and faster metabolic

alterations. There is no information on the rate of intestinal absorption of capsaicin. In the current investigation, the *in vitro* intestinal absorption of the three somewhat structurally closer bioactive spice principles – curcumin, capsaicin and piperine were evaluated using rat intestinal sections for making a comparison. In addition, the advantage of their encapsulation in phospholipid-bile salt micelles on the extent of the bioavailability of each one of these three spice principles in terms of intestinal absorption was evaluated.

In view of the observed dissimilarities in the *in vitro* absorption of curcumin, capsaicin and piperine in everted rat intestines, further studies were made in experimental animals to understand the bioavailability these compounds in terms of tissue distribution profile, and the rate of elimination following their oral administration. In the current investigation, studies were made on the *in vivo* tissue distribution and subsequent elimination following oral administration of curcumin, capsaicin and piperine to rats and the comparative results are presented in Section-B. Tissue distribution and elimination of curcumin orally administered concomitant with piperine was also examined in this investigation.

Since a number of animal studies indicate that these spice active principles reach the target tissues to exhibit their diverse physiological activities, it would be most relevant to understand how these essentially lipophilic compounds are transported in blood circulation to various tissues in the body following their intestinal absorption. Many lipophilic molecules like long-chain fatty acids are known to be carried by albumin in blood. The mode of transport of spice active compounds *in vivo* is not clear, except for limited studies in the case of curcumin. Spectroscopic studies on the interaction of curcumin with HSA has revealed that this compound interacts with HSA

at two binding sites, one with high affinity and the other with low affinity, and this binding does not change the conformation of HSA. Even though piperine and capsaicin have been understood to exhibit many physiological and pharmacological effects, their interaction with mammalian biopolymers such as HSA is almost unexplored. The affinity between ligand and HSA plays a crucial role in determining the bioavailability of many bioactive compounds such as drugs, phytonutrients, etc. In the present investigation, interaction of the spice principles - piperine and capsaicin with HSA has been examined employing spectroscopic measurements - fluorescence quenching and circular dichroism, size exclusion chromatography and Nuclear Magnetic Resonance. Such a study assumes importance in the context of understanding the carrier role of serum albumin for piperine and capsaicin transport in blood under physiological conditions.

The complex of enzymes present in the endoplasmic reticulum of mammalian cells known as Mixed Function Oxygenase System (MFOS) is involved in the hydroxylation of endogenous steroids besides its involvement in the primary metabolism of foreign compounds. A few spices are known to enhance catabolism of cholesterol to bile acids, which essentially involves hydroxylation of cholesterol by hepatic MFOS. Information on the influence of active principles of spices on MFOS might offer a possible explanation to their observed physiological effects related to metabolism of cholesterol, bile acids and steroid hormones. Since dietary antioxidant spices are now understood to protect against the induction of tumors by a variety of chemical carcinogens in experimental animals, which could be mediated via induction of detoxifying enzymes and Inhibition of chemical carcinogenesis may be brought about directly by scavenging the reactive substances or indirectly by promoting mechanisms

which enhance detoxification, it would be relevant to understand the influence of spice principles on hepatic MFOS. In this study, the effect of dietary supplementation of spice active principles - curcumin, capsaicin, combination of curcumin and capsaicin, and piperine on the activities of hepatic drug metabolizing enzymes was investigated. In the present study, these spice active principles were also examined for their possible *in vitro* influence on the components of hepatic MFOS in rat liver microsomal preparation.

Thus, the objectives of the present investigation were:

1) Evaluation of spices – turmeric (*Curcuma longa*), red pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) for the concentration of their active principles - curcumin, capsaicin and piperine during domestic food processing with variables such as presence of pulses and acidulants.
2) Structural characterization of the altered / degraded compounds arising from curcumin, capsaicin and piperine during heat processing.
3) Evaluation of the *in vitro* intestinal absorption of the three structurally related bioactive spice principles - curcumin, capsaicin and piperine using rat intestinal sections. Examination of the advantage of their encapsulation in phospholipid-bile salt micelles on the extent of their intestinal absorption.
4) Animal studies on the *in vivo* tissue distribution and subsequent elimination following oral administration of curcumin, capsaicin and piperine to rats.
5) Study of the interaction of the spice principles - piperine and capsaicin with Human Serum Albumin, employing spectroscopic measurements – fluorescence quenching and circular dichroism, size exclusion chromatography and NMR
6) Investigation of the effect of dietary supplementation of spice active principles - curcumin, capsaicin, combination of curcumin and capsaicin, and piperine on the activities of hepatic drug metabolizing enzymes. Examination of these compounds for their possible *in vitro* influence on the components of hepatic MFOS in rat liver microsomal preparation.

CHAPTER – II

EFFECT OF HEAT PROCESSING ON ACTIVE PRINCIPLES OF SPICES

Section - A: **Effect of heat processing of spices – turmeric red pepper and black pepper onthe concentrations of their bioactive principles**

Section - B: **Structural characterization of thermal degradation compounds of curcumin, capsaicin and piperine**

EFFECT OF HEAT PROCESSING ON ACTIVE PRINCIPLES OF SPICES

Introduction

Cooking or roasting alters the nature of many food constituents such as starches and proteins by changing their physical, chemical and nutritional characteristics (Belitz and Grosch, 1987). Heat processing also changes the bioavailability of proteins, carbohydrates, lipids and vitamins. Not much information is available on the extent of destruction of bioactive principles of spices during food processing. Spices are common food adjucts that impart flavour, aroma and colour to foods. In the present study, the effect of cooking process variables - temperature, duration, pH and presence of protein-rich staple grain on the extent of destruction of bioactive principles of turmeric (*Curcuma longa*), red pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) - the three most commonly used spices, has been evaluated.

Spices are extensively used as food adjuncts to enhance the sensory quality of foods. Among a host of beneficial physiological effects several spices are documented to exhibit, their hypolipidemic and antioxidant properties have far-reaching health implication. Work in our laboratory on the physiological effects of spices has focused on the influence on lipid metabolism, digestive stimulant action, beneficial influence of hypocholesterolemic spices on cholesterol gallstone disease and diabetic nephropathy, beneficial influence of antioxidant spice principles on inflammatory disease (Srinivasan, 2005).

Cooking or roasting in addition to altering physical, chemical and nutritional nature of starches and proteins, also changes the bioavailability of proteins, carbohydrates, lipids and vitamins. Since, many health beneficial physiological effects of spices are attributable to their active principles (Srinivasan, 2005a) there is a need to evaluate the availability of the spice active principles in their original form when spices are heat processed as in domestic cooking. Considerable extent of loss if any in the concentration of active principles would raise a question as to what extent the spices would retain their health beneficial effects after their conventional heat processing as in domestic food preparation. In this context, three spices - turmeric, red pepper and black pepper were evaluated for the status of their active principles during domestic food processing with variables such as presence of pulses and acidulants (Section-A).

Curcumin is a yellow compound (Fig.1A) isolated from the rhizomes of the turmeric plant (*Curcuma longa*). Turmeric is one of the main constituents of the spice mix - curry powder. Curcumin is a powerful anti-inflammatory, immunomodulating, tumor-preventing, and anti-atherogenic compound with a therapeutical potential (Miguel *et al*, 2002; Karunagaran *et al*, 2005). Curcumin is practically insoluble in water at acidic or neutral pH. At pH above neutral, dissociation takes place and the solubility increases, but the compound then undergoes rapid hydrolytic degradation (Tønnesen & Karlsen 1985; 1985a). Therefore, one of the challenges is to make a preparation with an acceptable curcumin solubility and stability at physiological pH. The pK_a values for the enol and the aromatic phenols have been determined to be 7.2, 8.5 and 9.0, respectively. The half-life for the hydrolytic degradation of curcumin in aqueous solution containing 10% organic solvent has previously been determined to be 4.2×10^3 h, 15 h and 3.5×10^{-2} h at pH 6, 7 and 8, respectively. A 50- to 500-fold increase

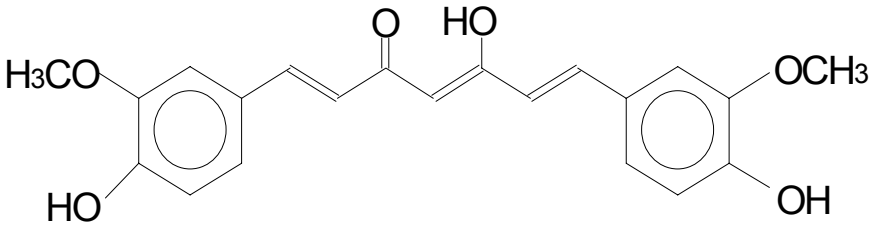
in stability can be obtained in aqueous media above pH 7 in the presence of various cyclodextrines (Tønnesen *et al,* 2002). Nearly an 1800-fold increase in stability can be obtained by addition of TX-100 to the solution (Tønnesen, 2002).

Capsaicin, the primary pungent principle (Fig.1B) in *Capsicum* fruits evokes numerous biological effects and thus has been the target of extensive investigations since its initial identification in 1919. One of the most recognized physiological properties of capsaicin is its selective effects on the peripheral part of the sensory nervous system, particularly on the primary afferent neurons of C-fibre type (Dray, 1992). Capsaicin has also been suggested for the initial management of neuralgia consequent to herpes infection (Bernstein 1989). Clinical trials revealed that topical cream containing 0.025% capsaicin significantly ameliorated the pain in patients with arthritis (Cordell & Araujo 1993). Extensive research in the last few years has shown that the pathway that activates nuclear transcription factor kappaB transcription factor can be interrupted by capsaicin (Aggarwal & Shishodia, 2004). Another field of intensive studies in recent times is diabetic neuropathy. Capsaicin has been shown to be useful in diabetic neuropathy (The Capasicin Study Group, 1992). Capsaicin, a dietary hypolipidemic spice principle of red pepper was found to be beneficial in protecting the structural integrity and fluidity of erythrocytes under conditions of hypercholesterolemia and hypetryglyceridemia (Kempaiah & Srinivasan, 2004). Capsaicin offer this beneficial effect by correcting the altered cholesterol to phosphorus ratio in the erythrocytes in both hypercholesterolemic and hypertryglyceridemic condition (Kempaiah & Srinivasan, 2004a). Dietary capsaicin has been observed to have a protective influence on induced oxidation of low-density lipoprotein in rats (Kempaiah *et al*, 2005).

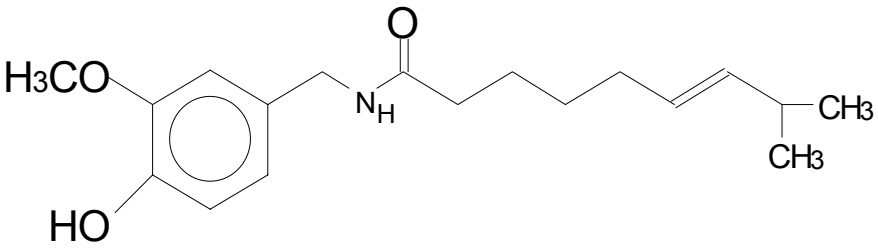
Piperine (1-Piperoyl piperidine) (Fig.1C) is the pungent alkaloid constituent of black and long peppers. Piperine has been reported as bioavailability modulator by several investigators (Shobha *et al*, 1998; Badmaev *et al*, 2000; Hiwale *et al*, 2002). The increased bioavailability is attributed to the effect of piperine on microsomal metabolizing enzymes or enzymes system. Khajuria *et al* (1998) have pointed out that piperine may act as an apolar molecule and form apolar complex with drugs and solutes. It may modulate membrane dynamics due to its easy partitioning thus helping in efficient permeability across the barriers. Khajuria et al (2000) also pointed out that piperine may be inducing alterations in membrane dynamics and permeation characteristics, along with induction in the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier. Piperine is reported to exhibit protective role against the oxidative alterations by carcinogens, growth-stimulatory activity towards cultured melanocytes (Khajuria et al, 1998a; Hoult et al, 1999). Dietary piperine, by favorably stimulating the digestive enzymes of pancreas, enhances digestive capacity and significantly reduces the gastro-intestinal food transit time (Platel & Srinivasan, 2004).

Since it was observed in this investigation that significant decrease in the concentrations of spice principles – curcumin, capsaicin and piperine would occur as a result of heat processing during domestic cooking processes such as boiling and pressure cooking, it would be relevant to assess the nature of altered / degraded compounds arising out of these parent spice compounds. Our studies also reveal that curcumin is more sensitive to heat compared to capsaicin and piperine. In this investigation, we have characterized some of the degraded compounds which are

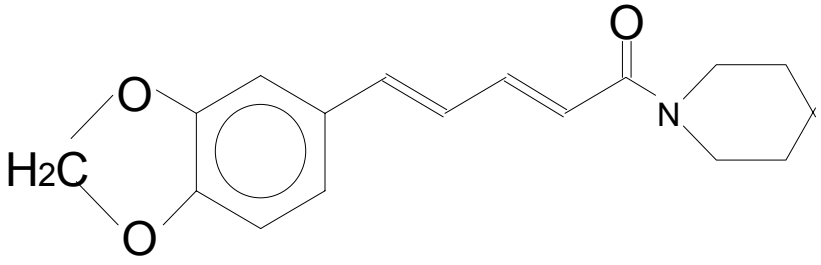
formed during the heat processing of spice principle – curcumin (Section-B). Studies were also made here in an effort to characterize the degradation products of capsaicin and piperine (Section-B). The present investigation assumes significance in the wake of the bioactivities of the degraded compounds which are formed during the heat treatment of curcumin the active principle of turmeric.



A. Curcumin



B. Capsaicin



C. Piperine

Fig. 1 Structures of curcumin, capsaicin and piperine, the active principles of the spices turmeric, red pepper and black pepper respectively

Section – A: Effect of heat processing of spices - turmeric, red pepper and black pepper on the concentrations of their bioactive principles

Summary

Studies were made to examine the loss of curcumin, capsaicin and piperine, the active principles of turmeric, red pepper and black pepper respectively as a result of subjecting the spices to domestic cooking process. This involved heat treatment of each of these spices by (i) boiling for 10 min, (ii) boiling for 20 min and (iii) pressure cooking for 10 min. Quantitation of the spice principles in the organic solvent extracts of the freeze-dried cooked spice samples was made with an appropriate HPLC method. Significant loss of spice active principles was observed when the spices were subjected to heat processing. Curcumin loss from heat processing of turmeric was 27 to 53%, with maximum loss in pressure cooking for 10 min. Curcumin loss from turmeric was similar even in presence of red gram. In presence of tamarind, the loss of Curcumin from turmeric was 12-30%. Capsaicin losses from red pepper were in the range 18 – 36% with maximum loss being observed in pressure cooking. Presence of either red gram or tamarind or both did not influence the loss of Capsaicin. Piperine losses from black pepper were in the range 16 – 34% with maximum loss being observed in pressure cooking. The loss was somewhat lower in presence of red gram. The results of this investigation indicated diminished availability of spice active principles from cooked foods, where the food ingredients would have been subjected to either boiling or pressure cooking for few minutes.

Materials and Methods

Materials

Spices were locally purchased and powdered to pass through No.50 mesh sieve. Tamarind (*Tamarindus indica*) dry pulp powder, decorticated red gram (*Cajanus cajan*) and common salt powder were from local market. Curcumin, capsaicin and piperine used as reference standard were procured from M/s. Fluka Chemie, Switzerland. Pepsin, pancreatin and bile extract were of porcine origin from M/s. Sigma Chemical Co., USA. All other chemicals used were of analytical grade and the solvents were distilled before use.

Heat treatment of spice samples

Studies were made to examine the loss of curcumin, capsaicin and piperine, the active principles of turmeric, red pepper and black pepper respectively as a result of subjecting the spices to cooking process at the domestic level. This involved heat treatment of each of these spices by (i) boiling for 10 min, (ii) boiling for 20 min and (iii) pressure cooking for 10 min at 15 p.s.i. The heat processing was done for the following samples, and was meant to simulate what is normally encountered in domestic cooking process: 1) Spice alone, 2) Spice + Red gram (decorticated), 3) Spice + Red gram (decorticated) + Tamarind, and 4) Spice + Tamarind. Precise quantity of individual spice powder (1g) was suspended in 100 ml water in parallel sets of 250 ml beaker. Tamarind powder (0.5 g) was added in 3rd and 4th set of samples. Addition of this acidulant brought down the pH by 1.0 unit. Portions of previously pressure-cooked (10 min) and mashed decorticated red gram equivalent to 5.0 g dry red gram was also added to the 2nd and 3rd set of samples. In the case of heat treatment by boiling, the

contents of the beaker were boiled exactly 10 or 20 min while taking care to maintain the volume around 100 ml by intermittent addition of water. At the end of heat treatment (boiling / pressure cooking), the samples were cooled to room temperature. Appropriate controls were also included wherein the samples did not undergo any treatment.

Spice samples in the lyophilized food samples were extracted with ethyl acetate in a Soxhlet apparatus for 4 h. The extracts were concentrated in a flash evaporator to a known volume (2 ml) and stored in dark at -20^{o}C until further analysis. The individual spice principles were quantitated after separation by TLC as described below. Care was taken to minimize the exposure to light during the extraction procedure and TLC separation. Quantitation of the spice principles in the organic solvent extracts of the freeze-dried cooked spice samples was also made with an appropriate HPLC method.

Quantitation of spice principles by TLC

Curcumin (Ravindranath *et al*, 1981)**:**

Aliquots of the ethyl acetate extracts (quadruplicate) of the lyophilized food material and reference curcumin (6 μg) were spotted on silica gel-G coated plates (20 X 20 cm). The plates were developed with the upper phase of the solvent system: benzene-ethanol-water-acetic acid (100 : 27.5 : 7.5 : 0.5 v/v/v/v) in a chamber pre-equilibrated with the above solvent system for 2 h. The yellow curcumin bands were scraped off and quantitatively transferred to centrifuge tubes. Curcumin in the scrapings was extracted with 4 ml acetone, centrifuged at 2000 rpm for 5 min and 2 ml of the clear supernatant was used for the rubrocurcumin reaction. Two ml of acetonic extract was transferred to another test tube into which were successively added, 1 ml 7.5 mg%

boric acid (in acetone) and 1 ml 5 mg% boric acid (in acetone). The contents of the tube were evaporated to dryness over a hot water bath. The residue was redissolved in 2 ml ethanol and absorption of the purple colour was measured at 550 nm.

Piperine (Bhat & Chandrasekhara, 1985)**:**

Aliquots of the ethyl acetate extracts of the food material were spotted on silica gel-G coated plates (20 X 20 cm) along with reference piperine (4 μg). Plates were developed with petroleum ether (60-80^{o}C) - acetone (65 : 35 v/v) in a chamber pre-equilibrated with the same solvent for 90 min. In order to locate the piperine bands, the developed plates were scanned in an automatic Camag TLC scanner (Model 2), mounted on a fluorimeter (Model 100, Turner Assoc.) attached to a W-W Recorder (Model III, Scientific instruments Inc.) The individual lanes were scanned using a primary filter No. 110-811 and a neutral density filter No. 110-813 (10%) as a secondary filter, and lamp No. 110-850 (Emission 310-390 nm). The piperine bands thus located were scraped off and eluted with 2 ml chloroform. After centrifugation the piperine extracts were quantified by absorption measurement at 345 nm.

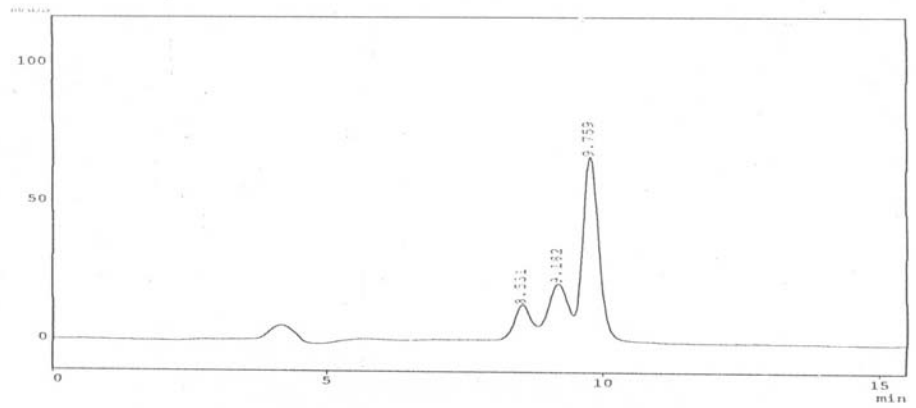
Capsaicin (Srinivasan *et al*, 1981)**:**

Aliquots of ethyl acetate extracts (quadruplicate) were spotted on silica gel-G coated plates (20 X 20 cm) along with reference capsaicin (40 μg). Plates were developed with petroleum ether (60-80^{o}C) - acetone (65 : 35 v/v) in a chamber pre-equilibrated with the same solvent for 90 min. The developed plates were air-dried and then sprayed uniformly with fresh Gibb`s reagent (0.1% 2,6-dichloroquinone-4-chlorimide in methanol). The plates, when dry, were exposed to ammonia vapours in a closed chamber for exactly 1 min. The blue-coloured zones of capsaicin thus

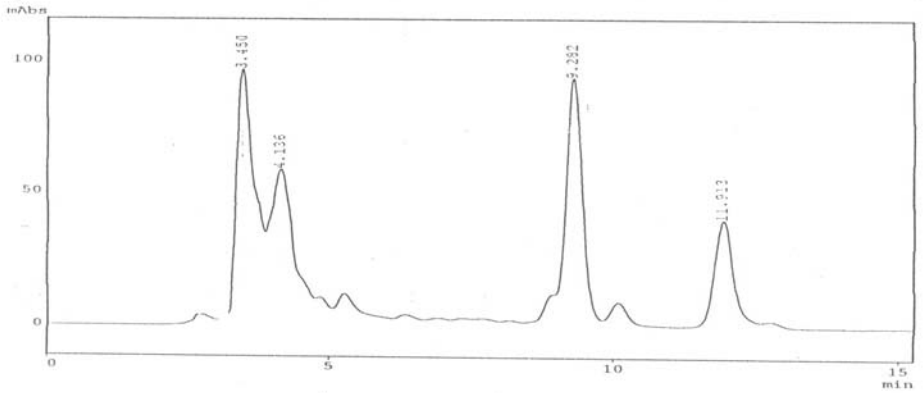
visualized were scraped off and quantitatively transferred to centrifuge tubes containing 2 ml water. The tubes were vortexed for 10 min to extract the colour and centrifuged at 2000 rpm for 2 min. Absorption of the clear blue supernatants was read at 610 nm.

Quantitation of spice principles by HPLC

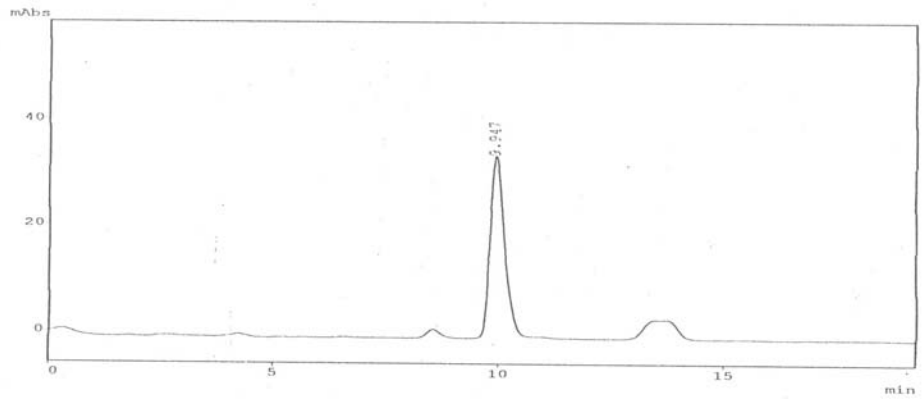
Quantitation of spice principles in the extracts cleaned up by passing through 2μ membrane filter was made by HPLC in a Shimadzu HPLC LC-10AT system consisting of a photo diode array detector, binary pump and manual sample injector. Chromatographic separation was accomplished using SGE 250 X 4.6 mm SSExcil C_{18} 10-μm column. Curcumin was analyzed by isocratic elution at 425 nm. Sample (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing acetonitrile-water-acetic acid (50:49:1 v/v/v) at a flow rate of 1 ml/min (Asai & Miyazawa, 2000). Quantitation of curcumin was made from peak area ratio, which was based on a calibration curve generated from standard curcumin. Piperine analysis was performed on reverse phase column with an isocratic elution at 345 nm (Bina, 1996). Ten μl piperine sample was eluted with a mobile phase containing a mixture of acetonitrile-water (50:50 v/v) at a flow rate of 1.0 ml/min. Quantitation of piperine was made from peak area ratio, which was based on a calibration curve generated from standard piperine. Capsaicin in the samples was separated and quantified by isocratic HPLC method using UV detection at a wavelength of 280 nm (Bina, 1996). An aliquot (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing a mixture of methanol-water (60:40 v/v) at a flow rate of 1.0 ml/min. Quantitation of capsaicin was made from peak area ratio, which was based on a calibration curve generated from standard capsaicin. HPLC elution profile of the three spice principles thus quantitated is given in Fig. 2.



Curcumin



Capsaicin



Piperine

Fig. 2. HPLC separation profile of active principles of spices

Simulated gastrointestinal digestion

Since there was a general under-estimation of spice principles in samples containing red gram, a supplementary study was carried out to digest the protein matrix of red gram by simulated gastrointestinal digestion procedure before extracting the spice principle (Miller et al, 1981) to verify if this would enhance the extractability of spice active principles. The specific samples containing the pulse - red gram were homogenized in additional amount of water; pH was adjusted to 2.0 with 6M HCl. Fresh pepsin solution (1.6 g pepsin suspended in 100 ml 0.1M HCl) was added (3 ml) and subjected to gastric digestion by incubation at 37°C for 2 h with shaking. Intestinal digestion was simulated by incubating for 3 h with shaking after raising the pH to 7.0 with 1M sodium hydroxide and addition of 5 ml freshly prepared pancreatin-bile mixture (4 g pancreatin + 25 g bile extract in 1L 0.1M sodium bicarbonate). Samples after simulated digestion as above were lyophilized and used for extraction of spice active principles.

Results

In this study, we have examined the loss of curcumin, capsaicin and piperine, the active principles of turmeric, red pepper and black pepper respectively as a result of subjecting the respective spices to domestic cooking process. This involved heat treatment of each of these spices by (i) boiling for 10 min, (ii) boiling for 20 min and (iii) pressure cooking for 10 min. Inclusion of tamarind at 0.5% level, brought down the pH of samples by one unit from 6.2 to 5.2. Inclusion of tamarind at 0.5% level brought down the pH of samples by one unit from 6.2 to 5.2.

Table 1. Concentration of spice principle - curcumin in heat-processed turmeric

Sample	Curcumin concentration (mg / g spice)			
	HP-0	HP-1	HP-2	HP-3
Turmeric	25.7 ± 1.44	18.8 ± 1.74 (27%)	17.3 ± 1.26 (32%)	12.1 ±1.74 (53%)
Turmeric + Red gram	9.25 ± 1.23	5.33 ± 0.33 (42.3%)	5.34 ± 0.55 (42.3%)	4.61 ± 0.68 (50.2%)
Turmeric + Red gram + Tamarind	8.74 ± 0.33	6.48 ± 0.83 (25.9%)	5.53 ± 0.29 (37%)	3.74 ± 0.14 (57.2%)
Turmeric + Tamarind	21.7 ± 0.15	18.5 ± 1.45 (14.7%)	17.0 ± 1.65 (21.7%)	14.3 ± 2.41 (34.3%)

Values are mean ± SEM of 5 independent determinations. Figures in parenthesis denote % loss.

HP-0 : Raw; HP-1 : Boiling for 10 min; HP-2 : Boiling for 20 min; HP-3 : Pressure cooking for 10 min at 15 lbs.

Table 2. Concentration of spice principle - capsaicin in heat-processed red pepper

Sample	Capsaicin concentration (mg / g spice)			
	HP-0	HP-1	HP-2	HP-3
Red pepper	2.932 ± 0.241	2.395 ± 0.183 (18.3%)	2.290 ± 0.160 (21.9%)	1.871 ± 0.174 (36.2%)
Red pepper + Red gram	1.173 ± 0.072	0.822 ± 0.062 (29.9%)	0.764 ± 0.055 (34.9%)	0.813 ± 0.068 (30.7%)
Red pepper + Red gram + Tamarind	1.201 ± 0.084	0.853 ± 0.091 (29.0%)	0.852 ± 0.099 (29.0%)	0.855 ± 0.071 (28.8%)
Red pepper + Tamarind	3.225 ± 0.252	2.432 ± 0.145 (24.6%)	2.445 ± 0.165 (24.2%)	2.167 ± 0.241 (32.8%)

Values are mean ± SEM of 5 independent determinations. Figures in parenthesis denote % loss.

HP-0 : Raw; HP-1 : Boiling for 10 min; HP-2 : Boiling for 20 min; HP-3 : Pressure cooking for 10 min at 15 lbs.

Table 3. Concentration of spice principle - piperine in heat-processed black pepper

Sample	Piperine concentration (mg / g spice)			
	HP-0	HP-1	HP-2	HP-3
Black pepper	55.8 ± 3.40	40.8 ± 2.76 (27.0%)	40.3 ± 3.26 (27.9%)	36.9 ± 2.74 (33.9%)
Black pepper + Red gram	12.8 ± 1.23	10.8 ± 0.86 (16.0%)	10.7 ± 0.85 (17.0%)	10.3 ± 0.78 (20.0%)
Black pepper + Red gram + Tamarind	13.3 ± 1.12	9.81 ± 0.83 (26.4%)	9.73 ± 0.64 (27.0%)	9.21 ± 0.64 (30.9%)
Black pepper + Tamarind	50.2 ± 4.15	41.2 ± 3.45 (18.0%)	39.7 ± 3.60 (21.0%)	33.8 ± 2.81 (32.7%)

Values are mean ± SEM of 5 independent determinations. Figures in parenthesis denote % loss.

HP-0 : Raw; HP-1 : Boiling for 10 min; HP-2 : Boiling for 20 min; HP-3 : Pressure cooking for 10 min at 15 lbs.

Significant loss of spice active principles as quantitated by HPLC procedure was observed when these three commonly used spices were subjected to such heat processing as in domestic cooking (Tables 1-3). Curcumin loss from heat processing of turmeric was 27 to 53%, with maximum loss in pressure cooking for 10 min (Table 1). Curcumin loss from turmeric was similar even in presence of red gram (42 - 50%) or red gram + tamarind (26 - 57%). In presence of tamarind, the loss of Curcumin from turmeric was comparatively lower (14-34%). Capsaicin losses from red pepper were in the range 18 – 36% with maximum loss being observed in pressure cooking (Table 2). In presence of either red gram or tamarind or both, the loss of capsaicin was in the range 25 - 35%. Thus, unlike curcumin, presence of the acidulant did not lower the loss of capsaicin during heat treatment. Piperine losses from black pepper were in the range 27 – 34% with maximum loss being observed in pressure cooking (Table 3). The loss was somewhat lower in presence of red gram (16 - 20%) or tamarind (18 - 33%). The pattern of changes in the concentration of curcumin, capsaicin and piperine upon heat processing of the parent spices was found to be similar when these compounds were quantitated by appropriate TLC procedures (Data not shown), although there were minor differences in the absolute values of spice principles between TLC procedure and HPLC procedure.

Discussion

Spices are common food adjuncts that are consumed in a variety of combinations depending on taste preferences. The present investigation was carried out to determine the extent to which the active principles of spices survive the domestic cooking treatments and finally remain in the food. The most common domestic methods of cooking are boiling the food ingredients in water and pressure-cooking

along with other food ingredients. pH variation was brought about by the presence and absence of tamarind, which is a common acidulant in Indian homes.

The present investigation has revealed that the loss of concentration of curcumin during domestic cooking procedure was maximum among the three spice principles, which have a somewhat similar structure with a common basic skeleton but different side chains (Fig. 1). A reduction of pH even by one unit has produced a considerable reduction in the loss of this active principle. It has been reported that curcumin in aqueous media is highly stable at pH below 7.0 at ambient temperature, while at pH > 7.0, curcumin is extremely unstable even at ambient temperature (Tonnesen & Karlsen, 1985). Alkaline degradation of curcumin has been reported to give ferulic acid and feruloyl methane, and that the feruloyl methane part of curcumin rapidly forms condensation products which are yellow to brownish yellow in colour (Tonnesen & Karlsen, 1985a). The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices have been recently investigated (Wang et al, 1997). When curcumin was incubated in 0.1 M phosphate buffer and serum-free medium, pH 7.2 at 37°C, about 90% decomposed within 30 min. A series of pH conditions ranging from 3 to 10 were tested and the result showed that decomposition was pH-dependent and occurred faster at neutral-basic conditions. It is more stable in cell culture medium containing 10% fetal calf serum and in human blood; less than 20% of curcumin decomposed within 1 h, and after incubation for 8 h, about 50% of curcumin still remained. Trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal was predicted as major degradation product and vanillin, ferulic acid, feruloyl methane were identified as minor degradation products.

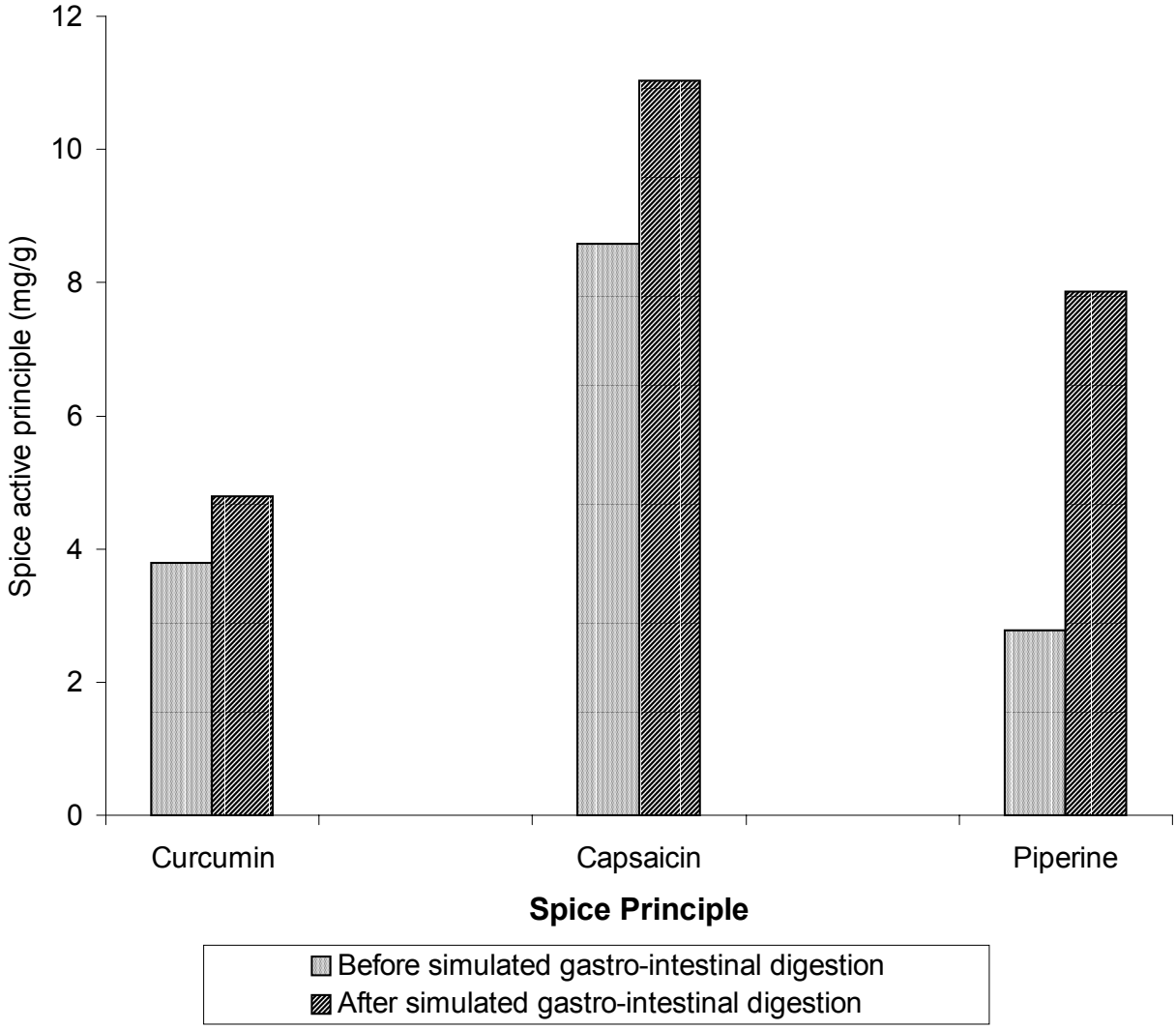


Fig. 3. Improved extractability of spice principles in presence of red gram after simulated gastro-intestinal digestion

There is no information about the stability of curcumin in aqueous solutions at higher temperatures, except for our earlier report (Srinivasan et al, 1992). We have also observed a shift in the absorption maxima from 280 nm to 293 nm of capsaicin solution when the pH is changed from neutral to alkaline, the shift in absorption maxima could be attributable to deprotonation of hydroxyl group of capsacin in alkaline pH (Data not shown). The present investigation has been an unambiguous study in that improved methodology, namely HPLC procedures have been employed for the quantitation of spice principles - curcumin, capsaicin and piperine. Further both the presence and absence of red gram, a common pulse ingredient in Indian curry dishes has been examined in this context. The loss of curcumin which we have observed in the present study, is contrary to the general belief that curcumin may be stable in aqueous solutions at pH either neutral or acidic.

Generally, there was an under-estimation of all the three spice principles in the case of samples containing red gram. This could be due to reduced extractability of the spice principle due to high affinity binding to proteins present in the pulse. Such a possibility was verified in a separate experiment by subjecting the food samples containing red gram to simulated gastrointestinal digestion following the heat treatment procedure and then extracting the respective spice principle with the organic solvent. As shown in Fig. 3, higher concentrations of each of these three spice principles was evidenced in samples subjected to simulated gastrointestinal digestion, which would have digested the protein present in the sample, thus facilitating extractability of the spice principle. The extractability of piperine was improved by 2.5-fold after simulated gastrointestinal digestion.

Conclusions

The results of this investigation indicated diminished availability of spice active principles from cooked foods, where the food ingredients would have been subjected to either boiling or pressure cooking for few minutes. Spices – turmeric, red pepper and black pepper are considered to be nutraceuticals due to multi-beneficial physiological effects they offer especially attributable their active principles (Srinivasan, 2005). In view of the significant extent of chemical alteration occurring to the spice active principles of turmeric and red pepper, it is desirable to determine the extent to which turmeric and red pepper retain their hypolipidemic and antioxidant potency upon thermal processing as in domestic cooking. Identification of the degradation products / altered products of spice principles - curcumin, capsaicin and piperine, arising out of heat processing of the respective parent spices is in progress.

Section – B: Structural characterization of thermal degradation compounds of curcumin, capsaicin and piperine

Summary

In the present study, we have attempted to understand the nature of altered / degraded compounds formed from spice active principles – curcumin, capsaicin and piperine as a result of heat treatment. Attempts were also made to structurally characterize the degradation products which are formed when curcumin / capsaicin / piperine was subjected to heat treatment. Three of the degraded compounds of curcumin were characterized as ferulic acid, vanillin and vanillic acid; while a few others could not be characterized. The UV absorption, proton NMR spectra and LC – MS/MS data of the three identified compounds matched very well with those of respective standards. Our studies confirm the vulnerability of the diketone bridge of curcumin molecule to heat. In addition, formation of vanillic acid and vanillin indicates that the molecule is sensitive to heat at the first carbon atom of the alkyl chain which is connecting the two benzene rings. Although capsaicin and piperine were understood to undergo degradation as a result of heat treatment, the compounds derived from these respective molecules could not be structurally characterized due to experimental limitations.

Materials and Methods

Chemicals

Curcumin, capsaicin and piperine were purchased from M/s Fluka Chemicals Co., Sweden, M/s. Sigma Chemicals Co., USA and M/s. Aldrich Chemicals Co., USA,

respectively. HPLC grade solvents were purchased from M/s. Ranbaxy Chemicals Co., New Delhi, India. Anhydrous Na_2SO_4 and other solvents were of analytical grade. All the solvents were distilled before use.

Sample preparation

Curcumin solution (0.1%) was prepared by dissolving in ethanol followed by the addition of water to suspend the compound such that the ethanol concentration does not exceed 1%. The curcumin suspension was subjected to heat treatment in a pressure cooker (15 p.s.i.) for 10 min. The heat-treated sample was extracted with ethyl acetate, dried over anhydrous Na_2SO_4 and concentrated by flash evaporation. The ethyl acetate extract was filtered through 0.45 μm Millipore filter, and used for chromatographic analysis.

Absorbance measurements

All the absorbance measurements were made at room temperature using a 1601 Shimadzu double beam UV spectrophotometer in a 1 cm path length cell.

Column Chromatography

The commercial curcumin sample which contains other curcuminoids - demethoxy curcumin and bis-demethoxy curcumin as contaminants was purified by employing column chromatography. The sample was specifically eluted from a column containing Silica gel (80 – 120 mesh) with benzene : ethyl acetate (98:2 v/v) to get pure curcumin. Demethoxy curcumin and bis-demethoxy curcumin are eluted with benzene : ethyl acetate in the ratio 95:5 and 90:10 v/v respectively.

Thin layer chromatography

Aliquots of the ethyl acetate extracts of the heat processed samples along with native (unheated) standards of active principles were spotted on silica gel-G coated (thickness: 300 mμ) analytical glass plates (20 X 20 cm). The plates were developed with chloroform : methanol (98:2 v/v) in a chamber pre-equilibrated with the same solvent system for 2 h. The developed plates were air-dried and exposed to iodine vapors in a closed chamber for exactly 3 min.

High Performance Liquid Chromatography

The Shimadzu HPLC LC-10AT system consisted of a photo diode array detector, binary pump and manual sample injector. Chromatographic separation of curcumin derived compounds was accomplished using Phenomenex 250 X 4.6 mm, 5 μm particle size ODS column. The mixture was eluted with a gradient mobile phase consisting of 20% of methanol and 80% of 1% acetic acid in water for first 35 min and the methanol concentration reached 100% in next 35 min. The major peaks were collected, dried over anhydrous Na_2SO_4 and concentrated by flash evaporator.

GC-MS

The degraded compounds of capsaicin and piperine were separated on SPB-1 capillary column with a temperature program from 60^{O} to 260 ^{O}C in 60 min on Perkin Elmer Gas chromatograph / Mass spectrometer instrument. Injector temperature was 240 ^{O}C and detector was set at 260 ^{O}C. The interface temperature was 250 ^{0}C. The mass spectra were recorded under electron impact ionization at 70 eV electron energy.

LC-MS / MS

Mass spectra of the compounds of the selected HPLC fractions were obtained using a Q-TOF Waters Ultima instrument fitted with an electron spray ionization (ESI) source. The data acquisition software - version 4.0 was used. The spectra were recorded in positive ion mode using spray voltage of 3.5 kV and a source temperature of 120 ^{0}C. Samples were prepared in the concentration range of 0.5 mg/mL in acetonitrile and injected by automated flow injector at a flow rate of 10 µL/min. Mass of the sample was recorded in the range of 100 – 1000.

NMR Spectroscopy

A Bruker DRX-500 MHz spectrometer operating at 500.13 MHz was used to record the ^{1}H NMR spectra in DMSO-d_6 with 100 µg of the sample dissolved in 50 µL of the solvent. About 50-200 scans were accumulated with a recycle period of 2-3 seconds to obtain a good spectrum with a 1 mm micro probe. The spectra were recorded at 35^{0} C with tetra methyl silane (TMS) as internal standard for measuring the chemical shift values to be within $\pm$ 0.001 ppm. A region from 0-10 ppm was scanned for all the samples.

Results and Discussion

Curcumin

Curcumin consists of two feruloyl moieties connected by a methylene bridge. It is well documented that, due to its β - diketone moiety, curcumin exists entirely in the enol form with a *trans* – geometry both in solid and solution (Tonnesen *et al,* 1982). Earlier investigations (Tønnesen, 2002; Tønnesen *et al*, 2002) have revealed that the

vulnerable part for of curcumin for hydrolysis and photolysis is the diketone bridge. This lead to the formation of several compounds such as feruloyl methane, ferulic acid, vanillin etc.

Several yellow spots (numbering 5 - 6) were observed upon exposure of the developed TLC plates of heat treated curcumin samples to iodine vapors. The native (unheated) curcumin gave 3 yellow spots which corresponded to curcumin, demethoxy curcumin and bis-demethoxy curcumin as expected. This suggested that the heat treatment of curcumin must have resulted in the formation of several curcumin derived compounds.

Due to the limitation in the resolution of the various newly formed compounds from heat treated curcumin (ethyl acetate extract), the sample was subjected to an appropriate HPLC method. When the HPLC elution profile of the heat treated curcumin sample was monitored at 425 nm (which is the absorption maximum of curcumin), there were no additional peaks corresponding to possible curcumin derived compounds. However, a decrease in the area under the peak corresponding to curcumin was observed in the case of heat-treated curcumin sample when compared to raw curcumin sample similarly subjected to HPLC analysis.

When the HPLC elution profile of the heat treated suspension of curcumin was monitored at 280 nm, several peaks in addition to the parent compound were observed (Fig.4). The same peaks excluding curcumin did not appear when the ethyl acetate extract of unprocessed curcumin suspension was chromatographed. Thus, the HPLC analysis indicates the formation of several curcumin derived compounds during the heat treatment.

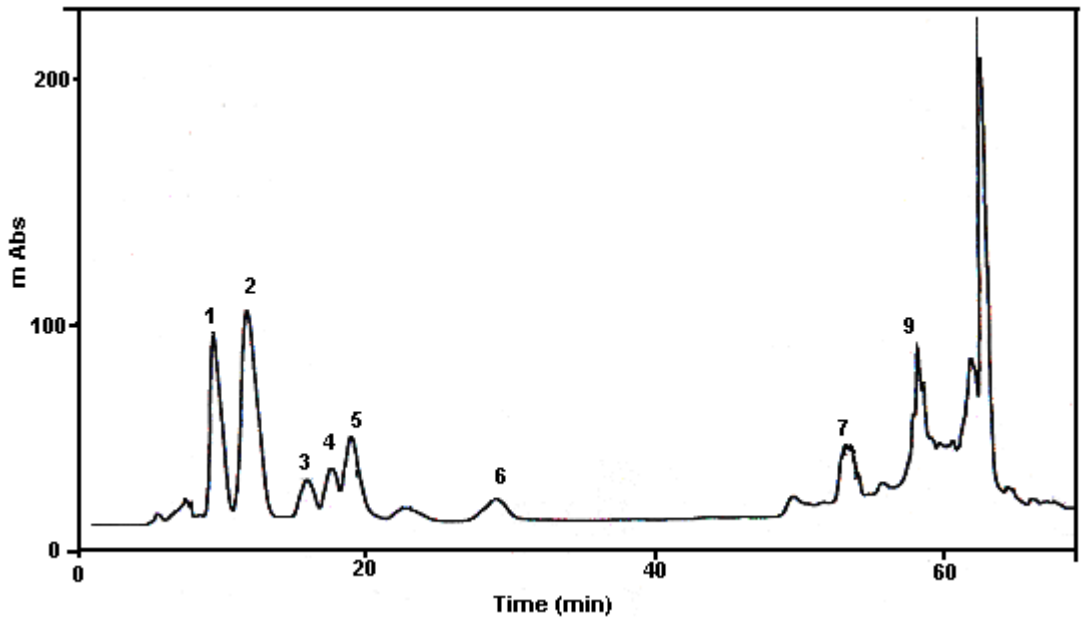


Fig. 4 HPLC profile of heat treated curcumin at 280 nm

The HPLC profile also indicates that the newly formed compounds from curcumin are not just out of minor modification of the parent compound, in which case, they would have retained the absorption profile of curcumin. The modification happening to curcumin could be of three types. (i) Minor modification in which the conjugated double bonds of curcumin may have got saturated leading to a blue shift in the absorption maxima of curcumin. (ii) Polymerization of curcumin, which will also lead to the change in the absorption profile of curcumin. (iii) Degradation of curcumin to smaller molecules. i.e. Formation of compounds having lower molecular weight. This will invariably alter the pattern of the curcumin absorption spectrum. The HPLC profile depicts the formation of several compounds which may be degraded compounds and/or altered compounds.

The HPLC fractions corresponding to peaks 1, 2 and 6 (Fig.4) were individually collected several times after repeated injections of the sample and pooled for further analysis. The other peaks (3,4,5,7,8, etc) obtained in this HPLC method were not collected for further analysis due to their poor resolution and hence the possibility of impurity. Structural characterization of the compounds in the collected fractions (1,2 and 6) was made by subjecting these HPLC fraction samples to NMR and mass spectral analysis. Figures 5, 6 and 7 depict the ^{1}H NMR spectra of the HPLC fractions 1, 2 and 6, respectively. These spectra resemble the ^{1}H NMR spectra of vanillic acid, ferulic acid and vanillin, respectively. This NMR study confirms the formation of these smaller compounds when curcumin is subjected to heat processing.

Further confirmation of the same was obtained from mass spectral analysis of the respective HPLC fractions. Fig. 8 represents the LC-MS spectrum of the HPLC-

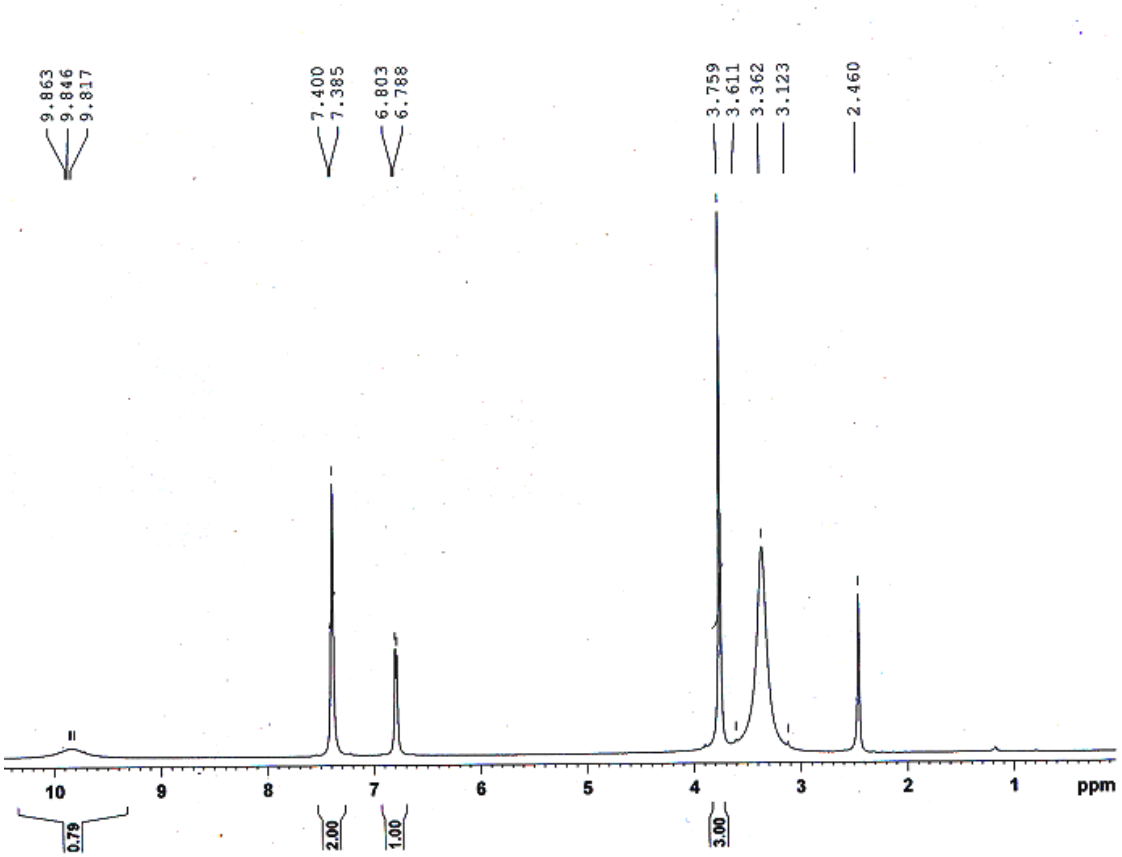


Fig. 5 ^{1}H NMR spectrum of HPLC fraction - 1 of heat processed curcumin

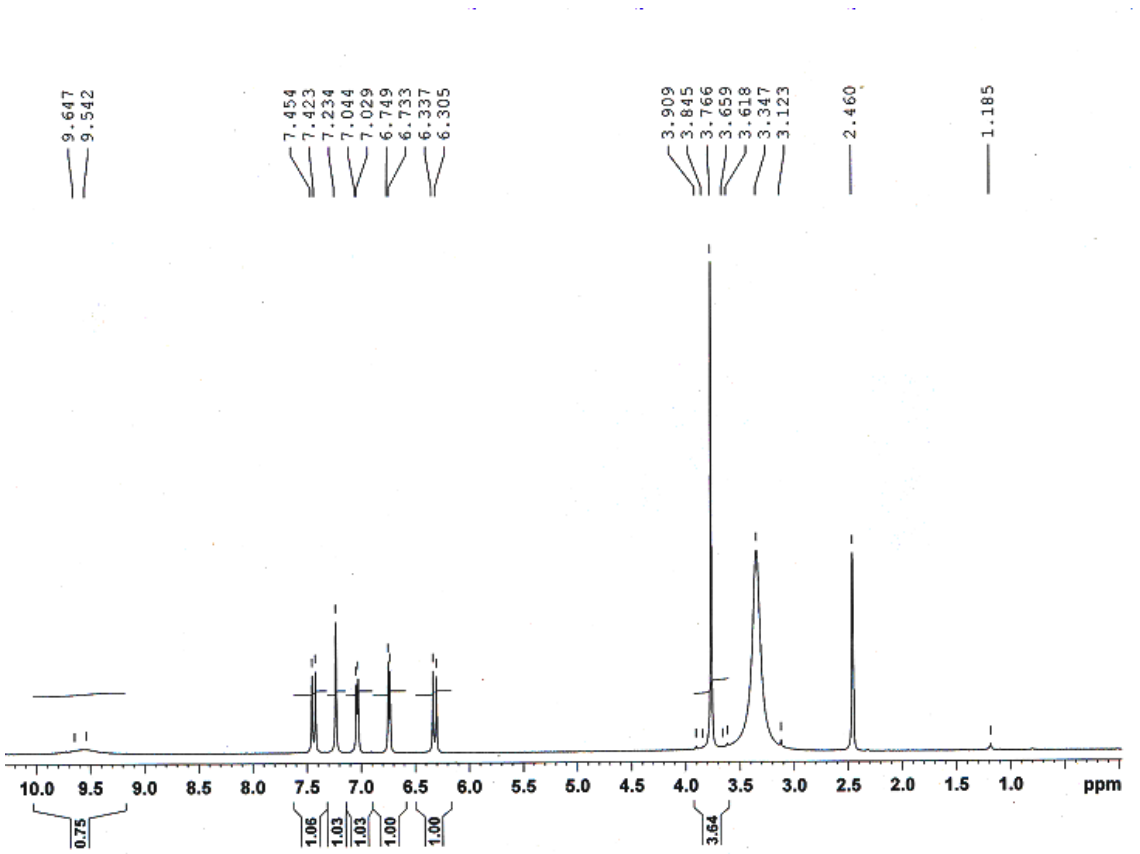


Fig. 6 ^{1}H NMR spectrum of HPLC fraction - 2 of heat processed curcumin

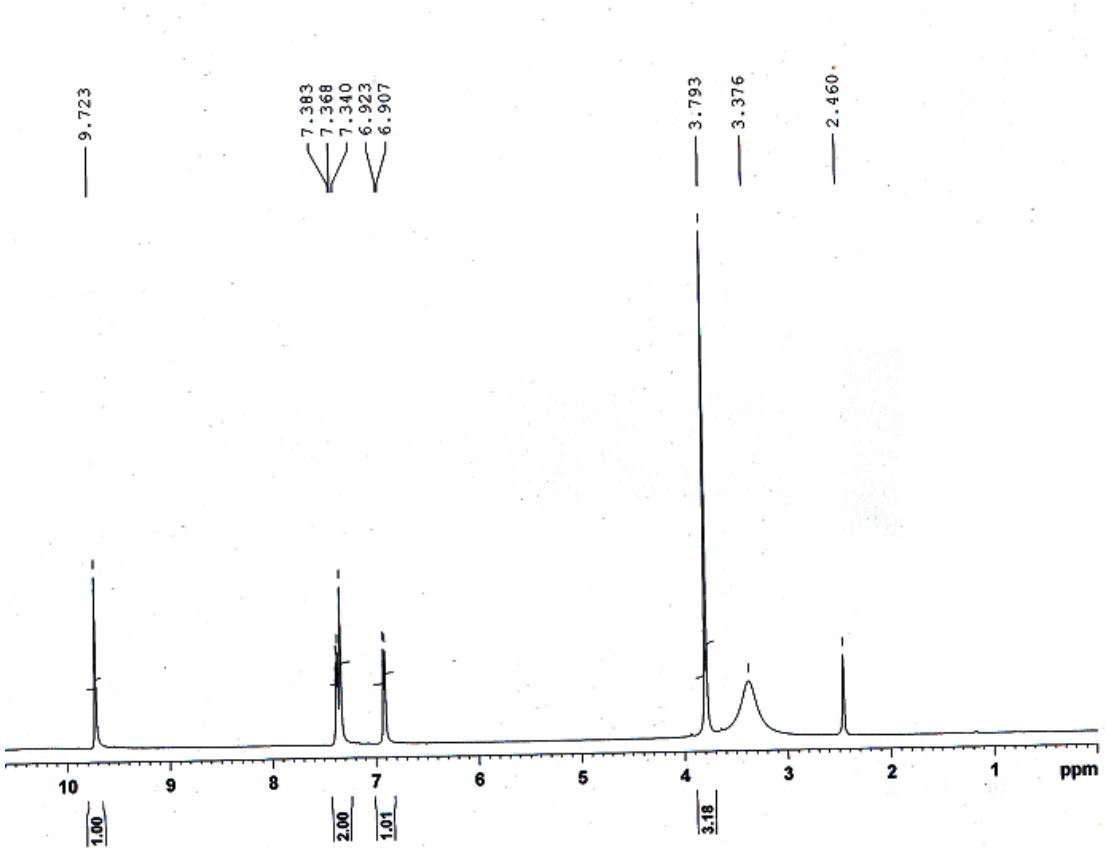


Fig. 7 ^{1}H NMR spectrum of HPLC fraction - 6 of heat processed curcumin

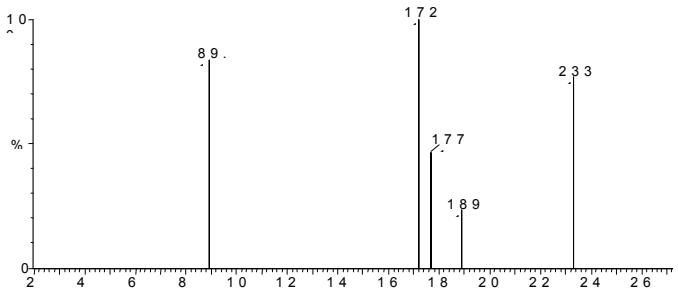


Fig. 8 LC-MS spectrum of the HPLC-fraction – 1 of heat processed curcumin

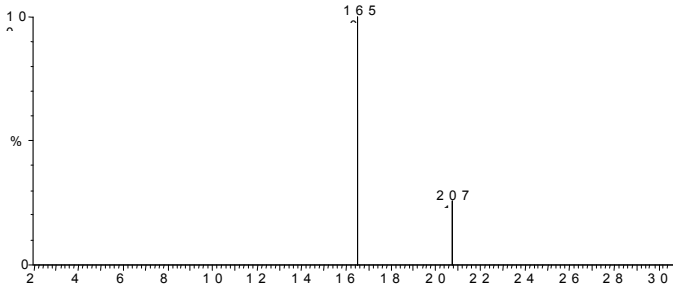


Fig. 9 LC-MS spectrum of the HPLC-fraction – 2 of heat processed curcumin

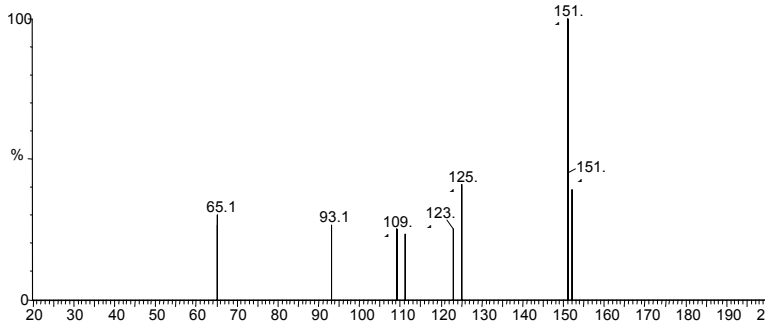


Fig. 10 LC-MS spectrum of the HPLC-fraction – 6 of heat processed curcumin

fraction 1. The molecular ion peak at 165 is due to the formation of $(M-3)^+$ ion of vanillic acid. Like wise, in Fig. 9 pertaining to LC-MS spectra of HPLC-fraction 2, the ion peak at 189 is due to the formation of $(M-5)^+$ of ferulic acid. In Fig.10, which presents the LC-MS spectra of HPLC-fraction 6, the molecular ion peak at 151 is due to $(M-1)^+$ ion of vanillin.

Thus, in the present study, we have characterized the structure of three degradation products among the several which are formed when curcumin is subjected to heat treatment. These compounds are ferulic acid, vanillin and vanillic acid. The UV absorption, proton NMR spectra and LC-MS/MS data of the three compounds match very well with those of respective standards. Our studies confirm the vulnerability of the diketone bridge in curcumin molecule to heat. In addition, formation of vanillic acid and vanillin indicates that the molecule is sensitive to heat at the first carbon atom of the alkyl chain which is connecting the two benzene rings. Structural identity of the other degradation compounds of curcumin could not be attempted in this study due to the limitations indicated earlier. Further HPLC studies involving advanced columns are necessitated to complete this task.

Capsaicin

Capsaicin is a member of the capsaicinoid family of chemosensory molecules found in *Capsicum* fruits. All capsaicinoids are vanillylamides of fatty acids that differ in the length of their aliphatic chain (Krajewska & Powers, 1988). The capsaicin hydrocarbon group consists of an 8-methyl non-6-enamide moiety that exists purely in the *trans* configuration about the double bond (Johnston *et al*, 2002). Capsaicin is an amphiphilic compound and as such it can associate with both hydrophilic and

hydrophobic groups. Capsaicin is sparsely soluble in water (60 mg/L) (Turgut *et al*, 2004) and thus it is expected to show little affinity for an aqueous phase.

When heat processed capsaicin sample was subjected to TLC and compared with that of unheated capsaicin, two extra spots were found in addition to the parent capsaicin spot. This indicates the thermal instability of capsaicin. These two additional spots which appeared on the chromatogram of the heat treated capsaicin sample may contain more than one compound each.

Due to this probable insufficient resolution, and also due to the volatile nature of the capsaicin, the heat treated sample was also subjected to gas chromatographic analysis. Fig.11 presents the gas chromatogram of the ethyl acetate extract of heat treated suspension of capsaicin. The GC profile of the heat treated capsaicin unambiguously indicated the presence of several compounds (numbering 5 - 6) in the heat treated capsaicin suspension.

Attempts were made to characterize the capsaicin derived compounds by subjecting the ethyl acetate extract of heat treated capsaicin sample to mass spectral analysis. However, this effort repeatedly made was not successful due to the encountering of contamination; Hence, the structure of the capsaicin derived compounds could not be obtained by mass spectral analysis.

Piperine

Piperine is a methylene dioxy phenyl compound containing a long hydrocarbon chain bearing a carbonyl group and is amide in nature. This is virtually insoluble in water.

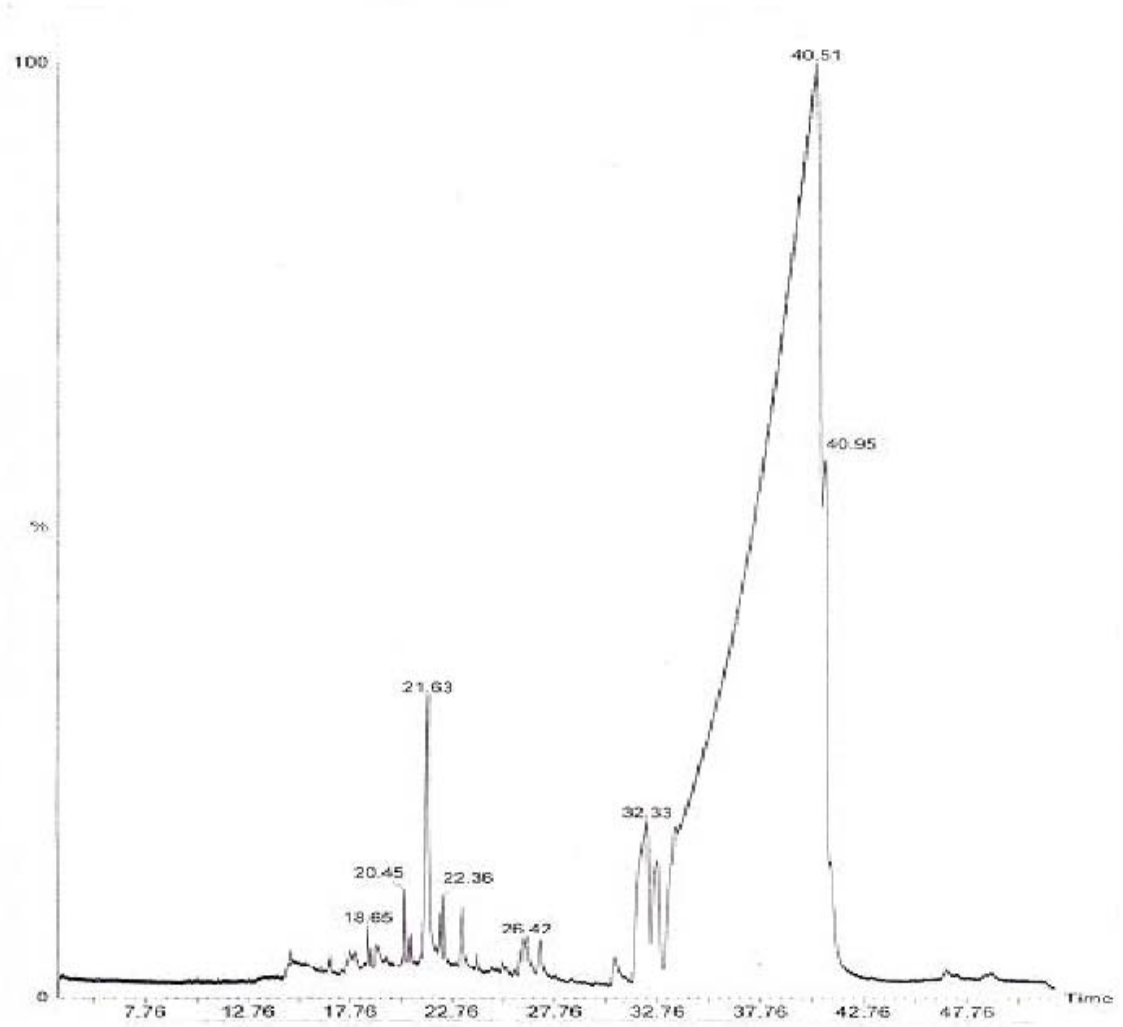


Fig. 11 GC profile of heat processed capsaicin

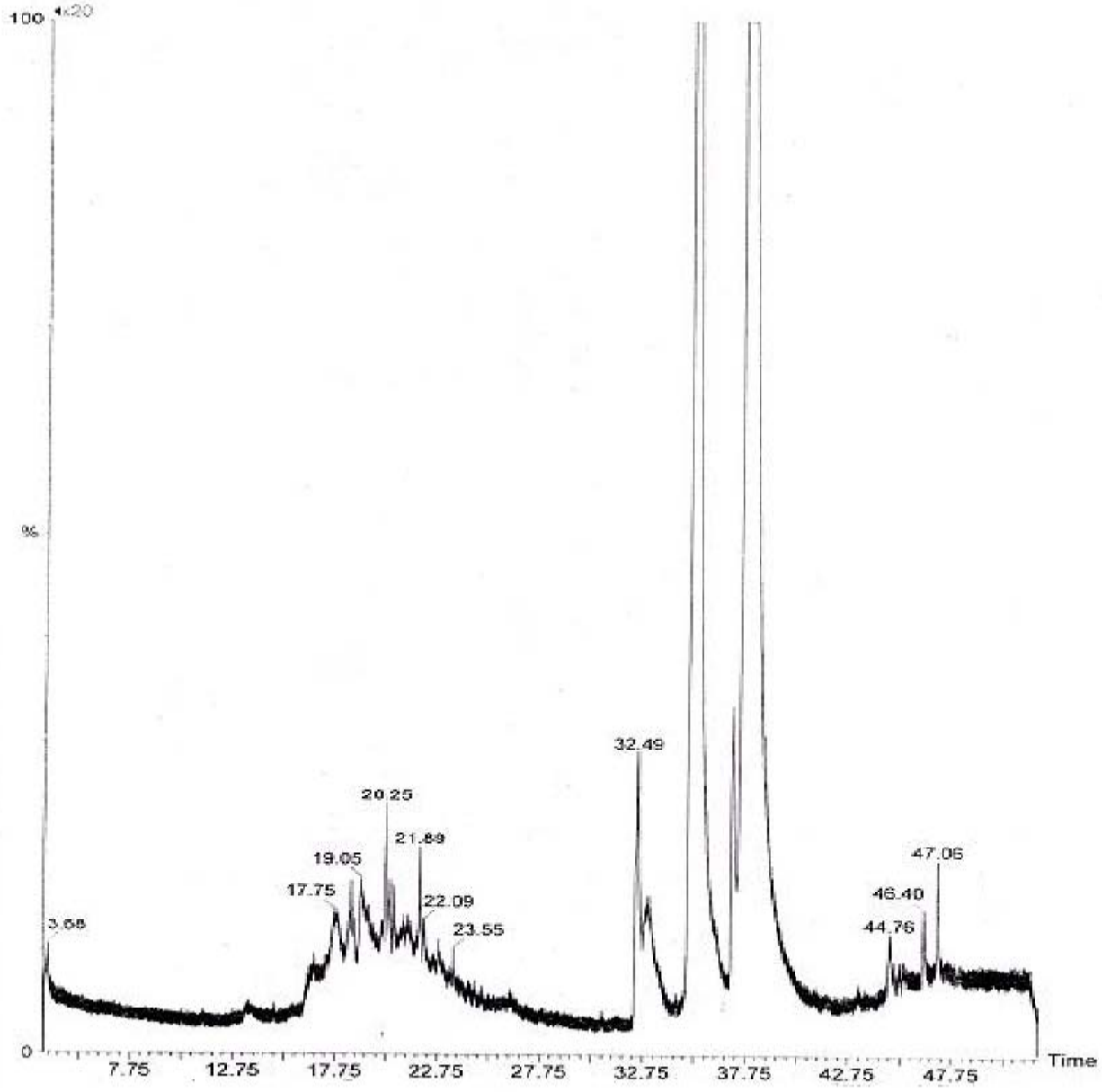


Fig. 12 GC profile of heat processed piperine

TLC profile of the heat treated piperine sample showed 6 – 8 additional spots compared to the TLC profile of raw piperine. This indicated the vulnerability of piperine to heat processing. Due to the ambiguity in the resolution of the spots in the thin layer chromatogram, the heat treated piperine sample was further subjected to gas chromatographic analysis. Fig.12 depicts the GC profile of heat treated piperine. The gas chromatographic analysis of the sample indicated the presence of several piperine derived compounds (numbering 8 - 9) in the heat processed sample. The same ethyl extract of the sample was subjected to mass spectral analysis for the characterization of the piperine derived compounds. As in the case of capsaicin, due to the problem of contamination, structural characterization could not be successfully completed for the newly formed compounds from piperine.

Conclusions

In the present study, we have attempted to understand the nature of altered / degraded compounds formed from spice active principles – curcumin, capsaicin and piperine. Attempts were also made to structurally characterize the degradation products which are formed when curcumin / capsaicin / piperine was subjected to heat treatment. Three of the degraded compounds of curcumin were characterized as ferulic acid, vanillin and vanillic acid; while a few others could not be characterized. The UV absorption, proton NMR spectra and LC – MS/MS data of the three identified compounds matched very well with those of respective standards. Our studies confirm the vulnerability of the diketone bridge of curcumin molecule to heat. In addition, formation of vanillic acid and vanillin indicates that the molecule is sensitive to heat at the first carbon atom of the alkyl chain which is connecting the two benzene rings. Although capsaicin and piperine were understood to undergo degradation as a result of

heat treatment, the compounds derived from these respective molecules could not be structurally characterized due to experimental limitations.

CHAPTER-III

PHARMACOKINETICS OF SPICE ACTIVE PRINCIPLES – CURCUMIN, CAPSAICIN AND PIPERINE

SECTION - A: Studies on the *in vitro* absorption of spice principles - Curcumin, Capsaicin and Piperine in rat intestines

SECTION - B: Studies on tissue distribution and elimination of orally administered spice principles – Curcumin, Capsaicin and Piperine in rats

PHARMACOKINETICS OF SPICE ACTIVE PRINCIPLES – CURCUMIN, CAPSAICIN AND PIPERINE

Introduction

Spices are documented to exhibit a host of beneficial physiological effects, among which the hypolipidemic and antioxidant properties have far-reaching health implication (Srinivasan, 2005). The health beneficial physiological effects of turmeric (*Curcuma longa*), red pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) are incidentally attributable to their respective active principles - curcumin, capsaicin and piperine (Srinivasan, 2005). These three spice principles share a considerable amount of structural homology. Curcumin (Diferuloyl methane) contains two ferulic acid moieties joined by a methylene bridge. Capsaicin is 8-methyl-N-vanillyl-6-nonanamide. Piperine is the *trans-trans* isomer of 1-piperoyl piperidine.

Curcumin is a potent antioxidant by virtue of which it is also anti-inflammatory and has anti-carcinogenic potential (Srinivasan, 2005). It exerts chemopreventive property in a wide variety of rodent models of carcinogenesis. Curcumin is a good scavenger of reactive oxygen species (Elizabeth & Rao, 1990) and inhibitor of lipid peroxidation (Reddy & Lokesh, 1994). It exerts chemopreventive property in a wide variety of rodent models of carcinogenesis (Kelloff *et al*, 1994). Curcumin lowers the formation of inflammatory compounds such as prostaglandins and leukotrienes by inhibiting the arachidonic acid metabolism by lipoxygenase and cyclooxygenase enzyme systems (Huang *et al*, 1991). Curcurmin is also an effective hypolipidemic agent (Srinivasan *et al*, 2004) and possesses antilithogenic property (Hussain & Chandrasekhara, 1993 & 1994). Curcumin is also useful as an antidiabetic food adjunct (Srinivasan, 2005a).

Capsaicin of red pepper is endowed with hypolipidemic (Srinivasan *et al*, 2004) and antilithogenic properties (Hussain & Chandrasekhara, 1993). Antioxidant properties of capsaicin by virtue of its potential to quench oxygen free radicals and inhibit lipid peroxidation have been documented in animal studies (Reddy & Lokesh, 1992). Anti-inflammatory property of capsaicin has been evidenced in experimental animals (Joe & Lokesh, 1997). Capsaicin has also received considerable attention as a pain reliever (Deal, 1991). Piperine has been demonstrated in *in vitro* studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Srinivasan, 2006). Piperine treatment has also been evidenced to lower lipid peroxidation and beneficially influence cellular antioxidant status in a number of experimental situations of oxidative stress (Srinivasan, 2006). The most far-reaching attribute of piperine has been its inhibitory influence on enzymatic drug biotransforming reactions in liver, which results in enhancing the bioavailability of a number of therapeutic drugs as well as phytochemicals (Srinivasan, 2006). Piperine has also been found to possess anti-mutagenic and anti-tumour influences (Srinivasan, 2006).

We have made an observation that when spices are heat processed as in domestic cooking, a considerable extent of loss in the concentration of these three spice active principles would occur (Previous Chapter). Structural characterization of the altered / degraded compounds arising from these spice principles has also been made (Previous chapter). In view of these health beneficial physiological effects which curcumin, capsaicin and piperine are known to elicit, there is a need to evaluate the bioavailability of these spice active principles. Curcumin is understood to possess low systemic bioavailability (Ireson *et al*, 2001). This pharmacokinetic feature of curcumin, which has been observed in several species, is attributed to a generally poor

absorption and faster metabolic alterations (Ireson *et al*, 2001; Ireson *et al*, 2002). There is no information on the rate of intestinal absorption of capsaicin.

In the current investigation, the *in vitro* intestinal absorption of the three somewhat structurally closer bioactive spice principles – curcumin, capsaicin and piperine (Fig.1) were evaluated using rat intestinal sections for making a comparison (Section-A). In addition, the advantage of their encapsulation in phospholipid-bile salt micelles on the extent of the bioavailability of each one of these three spice principles in terms of intestinal absorption was evaluated.

We have observed dissimilarities in the *in vitro* absorption of curcumin, capsaicin and piperine in everted rat intestines (Section-A of this Chapter). It is most relevant to understand the bioavailability, also in terms of tissue distribution profile, and the rate of elimination following their oral administration. In the current investigation, studies were made on the *in vivo* tissue distribution and subsequent elimination following oral administration of curcumin, capsaicin and piperine to rats and the comparative results are presented in Section-B. Tissue distribution and elimination of curcumin orally administered concomitant with piperine was also examined in this investigation.

Section – A : Studies on the *in vitro* absorption of spice principles - Curcumin, capsaicin and piperine in rat intestines

Summary

A comparative evaluation of the absorbability of three structurally similar and physiologically active spice principles in an *in vitro* system consisting of everted rat intestinal sacs was made. When everted sacs of rat intestines were incubated with 50-1000 μg of curcumin in 10 ml incubation medium, absorption of the spice principle was maximum at 100 μg concentration. The amount of absorbed curcumin present in the serosal fluid was negligible. This and the comparatively lower recovery of the original compound suggested that curcumin to some extent undergoes a modification during absorption. For similar concentrations of added piperine, about 44 - 63 % of piperine disappeared from the mucosal side. Absorption of piperine which was maximum at 800 μg per 10 ml was about 63%. The absolute amounts of piperine absorbed in this *in vitro* system exceeded the amounts of curcumin. The absorbed piperine could be traced in both the serosal fluid and in the intestinal tissue, indicating that piperine did not undergo any metabolic change during the process of absorption. 7 - 12 % of the absorbed piperine was found in the serosal fluid. When everted sacs of rat intestines were incubated with 10-500 μg of capsaicin, a maximum of 82-88 % absorption could be seen in the lower concentrations, and the amount of absorbed capsaicin did not proportionately increase at higher concentrations. A relatively higher percentage of the absorbed capsaicin could be seen in the serosal fluid as compared to curcumin or piperine. When these spice active principles were associated with mixed micelles, their *in vitro* intestinal absorption was relatively higher. Curcumin absorption in everted

intestinal sac increased from 48.7 to 56.1% when the same was present in micelles. In the case of capsaicin and piperine, increase in absorption was 27.8% to 44.4% and 43.4% to 57.4% respectively, when they were present in micelles as compared to its native form.

Materials and Methods

The three spice active principles – curcumin, capsaicin and piperine were procured from M/s Fluka Chemie, Switzerland. Phosphatidyl choline and sodium deoxycholate were from M/s Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade and the solvents were distilled before use.

Preparation of micelles:

Mixed micelles were prepared using a mixture of phosphatidyl choline and sodium deoxycholate in the molar ratio 1:2. After the phosphatidyl choline and deoxycholate were solubilized in chloroform: methanol (2:1 v/v), the solvent was evaporated in a flash evaporator and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris-HCl, pH 7.4 by sonication for 5 min in a bath-type sonicator. The spice active principles were added to mixed micelles in a molar ratio of active principle: micelle of 1:6 and vortexed for 30 seconds.

In vitro intestinal absorption studies:

Male albino rats of the Wistar strain weighing 140-150 g were maintained for 1 week on a semi synthetic diet with free access to food and water. The rats were stunned and after laporatomy the intestine was excised. After thoroughly washing both inside and outside with 0.9% saline, it was everted and cut into sacs of 8 cm length. Each sac was filled with Krebs-Ringer phosphate buffer containing 10 mM glucose.

Absorption of the three spice active principles was examined by incubating aerobically, the everted rat intestinal segments in the same Krebs-Ringer phosphate buffer - 10 mM glucose medium (10 ml) placed in a series of 25 ml conical flask containing varying concentrations of the spice principle. The spice principles were added to this medium as a suspension in water (0.1 ml) containing 0.04% Tween-20. The flasks were aerated with 95% oxygen and 5% carbon dioxide mixture and maintained at 37°C in a Julabo shaking water bath for 3 h. At the end of incubation, the sacs were removed, the mucosal surface washed and serosal contents collected. The mucosal medium and serosal fluid from different incubations were extracted for the spice principle with ethyl acetate and the intestinal tissue from different incubations were extracted for the spice principle by Folch procedure with chloroform-methanol (2:1 v/v) and the extracts were analyzed for the concerned spice principle by an appropriate HPLC procedure.

Quantitation of spice principles by HPLC:

Quantitation of spice principles in the extracts cleaned up by passing through 2μ membrane filter was made by HPLC in a Shimadzu HPLC LC-10AT system consisting of a photo diode array detector, binary pump and manual sample injector. Chromatographic separation was accomplished using SGE 250 X 4.6 mm SS Excil C_{18} 10-μm column. Curcumin was analyzed by isocratic elution at 425 nm. Sample (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing acetonitrile-water-acetic acid (50:49:1 v/v/v) at a flow rate of 1 ml/min (Asai & Miyazawa, 2000). Quantitation of curcumin was made from peak area ratio, which was based on a calibration curve generated from standard curcumin. Piperine analysis was performed on reverse phase column with an isocratic elution at 345 nm (Bajad *et al*, 2002). Ten μl piperine sample was eluted with a mobile phase containing a mixture of

25 mM potassium dihydrogen phosphate (pH 4.5) and acetonitrile (35 : 65 v/v) at a flow rate of 1.0 ml/min. Quantitation of piperine was made from peak area ratio, which was based on a calibration curve generated from standard piperine. Capsaicin in the samples was separated and quantified by isocratic HPLC method using UV detection at a wavelength of 280 nm (Saria *et al*, 1981). An aliquot (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing a mixture of methanol-water (60:40 v/v) at a flow rate of 1.0 ml/min. Quantitation of capsaicin was made from peak area ratio, which was based on a calibration curve generated from standard capsaicin.

Results and Discussion

In vitro absorption of curcumin:

The amounts of curcumin present in the incubation medium and the extent of curcumin that got absorbed from the mucosal side into the sac tissue and then to the serosal side is indicated in Table-1. When everted sacs of rat intestines were incubated with 50 - 1000 μg of curcumin per 10 ml incubation medium, absorption of this spice principle by the everted intestinal sacs was proportionate only below 100 μg and reached maximum at 100 μg concentration. Recovery of added curcumin was around 79 - 83 %. At the lowest concentration of 50 μg per 10 ml incubation medium, 80.4 % of the added curcumin disappeared from the mucosal medium, and at 100 μg per 10 ml incubation medium, 83% of the added curcumin disappeared from the mucosal medium. When curcumin concentration in the incubation medium further increased, the % absorption proportionately decreased; about 40% of curcumin was absorbed when curcumin concentration was 1000 μg per 10 ml incubation medium. Further, at the end of 3-h incubation period, the amount of curcumin present in the

serosal fluid following absorption was strikingly negligible at any of the concentration levels.

The comparatively lower recovery of curcumin as well as its negligible amounts found in serosal fluid suggest that curcumin is undergoing a modification during absorption. About 10% of the curcumin added to the incubation medium that remained unaccounted in the serosal medium or the intestinal sac tissue, must have modified in its structure in the intestine so that it is not quantitated as curcumin. A similar observation has been reported in an earlier study, where the authors have employed thin layer chromatography for the quantitation of the spice principle and observed that no curcumin was detected in the serosal fluid at any of the concentrations of curcumin added to incubation medium (Vijayalakshmi & Chandrasekhara, 1981). Further, these researchers did not detect intact curcumin in the intestinal tissue after incubation in a medium containing up to 50 μg curcumin, while only about 2.5% of the added curcumin was present in the intestinal tissue even at the highest concentration of 750 μg per 10 ml of the incubation medium. However, in the present investigation we have detected intact curcumin in the serosal fluid after incubation of the intestinal sac at any of the varying concentrations of curcumin ranging from 50 to 1000 μg per 10 ml, although as a very small portion of the absorbed curcumin. Further, in the current study, the major portion of the absorbed curcumin was detected in the intestinal sac tissue and this was true at any of the concentrations of curcumin in the incubation medium examined. This deviation in the observation from the earlier report with regard to curcumin absorption

Table 1. *In vitro* absorption of curcumin by everted rat intestinal sac

Curcumin in mucosal medium (μg/10 ml)	Curcumin concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Sac tissue	Serosal fluid		
50	7.87 ± 0.65 (19.6%)	31.8 ± 3.13 (79.2%)	0.48 ± 0.05 (1.2%)	80.3	80.4
100	14.1 ± 0.59 (17.1%)	68.2 ± 3.13 (82.5%)	0.36 ± 0.04 (0.4%)	82.7	82.9
200	44.5 ± 2.23 (26.9%)	120.7 ± 12.2 (72.8%)	0.53 ± 0.05 (0.3%)	82.9	73.1
400	113.4 ± 10.5 (35.9%)	201.6 ± 13.5 (63.9%)	0.57 ± 0.07 (0.2%)	78.9	60.3
600	213.1 ± 4.89 (44.1%)	269.7 ± 8.67 (55.8%)	0.35 ± 0.03 (0.1%)	80.5	55.9
800	312.9 ± 13.3 (49.0%)	324.7 ± 9.44 (50.9%)	0.44 ± 0.04 (0.07%)	79.8	51.0
1000	494.5 ± 13.3 (60.0%)	329.7 ± 8.75 (40.0%)	0.37 ± 0.04 (0.04%)	82.5	40.0

Values are mean ± SEM of 5 independant samples

Values in parenthesis represent % distribution

from everted intestinal sacs can be attributed to the more sensitive HPLC method employed here for the determination of intact curcumin in the serosal fluid, intestinal tissue and mucosal medium.

In vitro absorption of piperine:

The relationship between the amounts of piperine in the incubation medium and the percentage of piperine that got absorbed from the mucosal side into the sac tissue and then to the serosal side is indicated in Table-2. For similar concentrations of added piperine, viz., 200 – 1000 μg per 10 ml incubation medium, about 44 - 63 % of the compound disappeared from the mucosal side. The absorbed piperine could be traced in the serosal fluid and in the intestinal tissue, indicating that piperine did not undergo any metabolic change during the process of absorption. Recovery of added piperine was about 87 - 90 %, which is significantly higher than that of curcumin under similar conditions. As much as 3.5 - 7.5 % of the added piperine in the incubation medium was found in the serosal fluid. This accounted for 7.1 – 11.8% of absorbed piperine. Whereas the extent of absorption of added piperine in the incubation medium was lesser than that of curcumin for similar concentrations up to 500 μg per 10 ml, piperine absorption continued to increase till piperine present was 800 μg of per 10 ml of incubation medium. A maximum 63% absorption of piperine was observed when it was present at 800 μg per 10 ml incubation medium. The absolute amounts of piperine absorbed in this *in vitro* system exceeded the amounts of curcumin when the concentration of the spice principle presented for absorption was 800 and 1000 μg per 10 ml incubation medium. The present observation of the absorption potential of piperine *in vitro* by everted rat intestinal segments is in agreement with an earlier report

Table 2. *In vitro* absorption of piperine by everted rat intestinal sac

Piperine in mucosal medium (μg/10 ml)	Piperine concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Sac tissue	Serosal fluid		
200	99.8 ± 8.6 (56.0%)	71.4 ± 2.3 (40.0%)	7.10 ± 0.74 (4.0%)	89.2	44.0
400	188.3 ± 7.1 (52.6%)	157.8 ± 4.7 (44.0%)	12.0 ± 0.47 (3.4%)	89.5	47.4
500	208.5 ± 10.6 (47.5%)	212.5 ± 9.2 (48.4%)	18.2 ± 0.52 (4.1%)	87.8	52.5
600	223.0 ± 12.0 (42.7%)	266.7 ± 6.1 (51.1%)	32.1 ± 2.10 (6.2%)	87.0	57.3
800	267.9 ± 14.5 (37.2%)	397.8 ± 8.3 (55.3%)	53.6 ± 3.10 (7.5%)	89.9	62.8
1000	380.4 ± 5.40 (42.2%)	477.2 ± 11.2 (53.0%)	42.8 ± 2.20 (4.8%)	90.0	57.8

Values are mean ± SEM of 5 independent samples

Values in parenthesis represent % distribution

by Bhat & Chandrasekhara (1986) who have reported 47 – 64% absorption of piperine present at a concentration of 200 – 1000 μg per 10 ml incubation medium.

In vitro absorption of capsaicin:

When everted sacs of rat intestines were incubated with 10 - 500 μg of capsaicin, a maximum of 82.5 - 88 % absorption could be seen in the lower concentrations, i.e., 10 -50 μg per 10 ml and the amount of absorbed capsaicin did not proportionately increase at further higher concentrations (Table-3). Absorption of capsaicin appeared to have reached a saturation point at 100 μg per 10 ml concentration of the spice principle in the incubation medium, after which point there was either no absorption of further amounts or it was only a marginal increase. A relatively much higher percentage of the absorbed capsaicin could be seen in the serosal fluid as compared to curcumin and piperine. Thirty to forty-five percent of absorbed capsaicin could be traced in serosal fluid. Recovery of added capsaicin was 82 - 87 % which is higher than that of curcumin. The data are suggestive that no modification of this spice principle occurs during its absorption. In terms of the absolute amounts of capsaicin that can get absorbed under similar conditions, intestinal absorption of capsaicin was least among the three spice principles examined in this study, and this was much lesser compared to the amounts of either curcumin or piperine that get absorbed for a given concentration presented for absorption. There are no previous reports on the intestinal absorption potential of capsaicin.

In vitro absorption of spice principles present in micelles:

A comparison of the absorption potential of curcumin, piperine and capsaicin presented for absorption in micellar form relative to the native form in the same set up

Table 3. *In vitro* absorption of capsaicin by everted rat intestinal sac

Capsaicin in mucosal medium (μg/10 ml)	Capsaicin concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Sac tissue	Serosal fluid		
10	1.03 ± 0.12 (12.1%)	4.59 ± 0.30 (54.0%)	2.88 ± 0.29 (33.9%)	85.0	87.9
25	2.89 ± 0.38 (14.0%)	9.62 ± 1.10 (46.8%)	8.06 ± 0.56 (39.2%)	82.3	86.0
50	7.42 ± 0.75 (17.5%)	20.5 ± 1.02 (48.3%)	14.5 ± 1.73 (34.2%)	84.8	82.5
100	27.5 ± 2.65 (32.1%)	40.7 ± 1.72 (47.5%)	17.4 ± 1.03 (20.3%)	85.6	67.8
250	156.9 ± 5.68 (73.1%)	35.9 ± 1.39 (16.7%)	21.9 ± 1.14 (10.2%)	85.9	26.9
500	355.0 ± 18.2 (81.2%)	50.7 ± 3.08 (11.6%)	31.4 ± 2.73 (7.18%)	87.4	18.8

Values are mean ± SEM of 5 independent samples

Values in parenthesis represent % distribution

Table 4. *In vitro* absorption of micellar curcumin in everted rat intestinal sac

	Curcumin concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Serosal fluid	Sac tissue		
Micellar Curcumin					
1 hr	429 ± 7.1 (66.7%)	1.18 ± 0.59 (0.2%)	213 ± 9.8 (33.1%)	80.4	33.3
2 hr	359 ± 8.2 (57.7%)	0.8 ± 0.10 (0.13%)	262 ± 8.0 (42.14%)	77.7	42.3
3 hr	273 ± 5.0 (43.9%)	0.69 ± 12.2 (0.11%)	348 ± 11.5 (56.0%)	77.7	56.1
Native Curcumin					
3 hr	332 ± 10.6 (51.3%)	0.46 ± 0.04 (0.07%)	315 ± 5.5 (48.7%)	80.9	48.7

Values are mean ± SEM of 5 independent samples

Values in parenthesis represent % distribution

Table 5. *In vitro* absorption of micellar piperine in everted rat intestinal sac

	Piperine concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Serosal fluid	Sac tissue		
Micellar Piperine					
1 hr	124 ± 5.1 (75.1%)	10.1 ± 1.2 (6.1%)	31 ± 5.2 (18.8%)	82.6	24.9
2 hr	109 ± 3.4 (64.0%)	16.4 ± 1.4 (9.62%)	45 ± 2.6 (26.4%)	85.2	36.0
3 hr	72 ± 2.5 (42.6%)	12.0 ± 2.5 (7.10%)	85 ± 2.5 (50.3%)	84.3	57.4
Native Piperine					
3 hr	102 ± 2.9 (56.6%)	8.3 ± 1.1 (4.6%)	70 ± 3.6 (38.8%)	90.6	43.4

Values are mean ± SEM of 5 independent samples

Values in parenthesis represent % distribution

Table-6 *In vitro* absorption of micellar capsaicin in everted rat intestinal sac

	Capsaicin concentration after incubation			Recovery %	Absorption %
	Mucosal fluid	Serosal fluid	Sac tissue		
Micellar Capsaicin					
1 hr	167 ± 3.3 (81.07%)	10 ± 1.0 (4.85%)	29 ± 3.0 (14.08%)	82.4	18.9
2 hr	133 ± 6.4 (65.2%)	25 ± 2.3 (12.3%)	46 ± 4.0 (22.5%)	81.6	34.8
3 hr	114 ± 3.1 (55.6%)	29 ± 4.2 (14.15%)	62 ± 5.2 (30.25%)	82.0	44.4
Native Capsaicin					
3 hr	156 ± 8.5 (72.2%)	25 ± 2.3 (11.6%)	35 ± 3.1 (16.2%)	86.4	27.8

Values are mean ± SEM of 5 independent samples

Values in parenthesis represent % distribution

of everted sac intestinal segments are presented in Tables 4 - 6. It was observed that curcumin absorption in everted intestinal sac increased from 48.7 to 56.1% when the same was present in micelles and added at a concentration of 800 μg per 10 ml of the incubation medium (Table-4). In the case of capsaicin, absorption was increased to 44.4% when capsaicin was present in micelles when compared to its native form (27.8%) when added at a concentration of 250 μg per 10 ml (Table-5). Similarly, in the case of piperine, absorption was increased to 57.4% from 43.4%, as a result of its presence in micelles and added at a concentration of 200 μg per 10 ml (Table-6). Thus the absorption of all the three spice principles was significantly enhanced for a given concentration of the same present in the incubation medium in the form of micelles. This observation probably has practical application in the use of these spice principles for pharmacological purposes.

Conclusions

The numerous biological effects attributable to the spice principles – curcumin, capsaicin and piperine indicate that these compounds could serve as functional food ingredients. Development of drugs out of such natural constituents requires information on pharmacokinetic and safety aspects. A comparative evaluation of the absorbability of three structurally similar and physiologically active spice principles in an *in vitro* system consisting of everted rat intestinal sacs was made. Among the three spice active principles examined for their absorption potential from the everted rat intestines, capsaicin is absorbed to a least extent for a given concentration of the spice principle. Only a minimum portion of the absorbed curcumin was traced in serosal fluid at the end of 3 h incubation period in the current *in vitro* study, while most of it was still

present in intestinal tissue. The absolute amounts of piperine absorbed in this *in vitro* system exceeded the amounts of curcumin. The relatively lesser recovery of curcumin suggested transformation of the compound during absorption thus explaining the reported low systemic availability of the original compound. The absorption of the spice principles was comparatively higher when these are present in the form of micelles.

Section – B: Studies on tissue distribution and elimination of orally administered spice principles – curcumin, capsaicin and piperine in rats

Summary

Tissue distribution and elimination of three structurally similar bioactive spice principles - curcumin, capsaicin and piperine following their oral intake was examined in rats. Distribution in blood, liver, kidney and intestine in 1 - 24 h was 1.24 – 24.4 % of administered dose for capsaicin, 3.1-10.8 % for piperine, and 3.88 – 48.3 % for curcumin. Maximum distribution of 24.4 % of administered capsaicin was seen at 1 h, while the same was not detectable after 4 days. Absorption of capsaicin was about 94% and very rapid relative to other two compounds. A maximum of 10.8 % of administered piperine was seen in tissues at 6 h. Absorption of the administered piperine was about 96%. Curcumin concentration was maximum in the intestine at 1h; maximum in blood at 6 h and remained at significantly higher level even at 24 h. About 63.5 % of the curcumin dose was absorbed. Only a small portion of the administered dose of capsaicin (< 0.1%) and curcumin (0.173 %) was excreted in urine, whereas piperine was not detectable in urine. Enhanced bioavailability of curcumin was evidenced when the same was orally administered concomitant with piperine. Intestinal absorption of curcumin was relatively higher, and it stayed significantly longer in the body tissues. Intact curcumin was detected in brain at 24, 48 and 96 h with a maximum at 48 h.

Methods and Materials

The three spice active principles – curcumin, capsaicin and piperine were procured from M/s Fluka Chemie, Switzerland. All other chemicals used were of analytical grade and the solvents were distilled before use.

Animal treatment:

Experiments on animals were conducted with due approval of the procedures by the Institute's Animal Ethics Committee with regard to the care and use of animals. *In vivo* tissue distribution and excretion studies with spice principles – curcumin, capsaicin and piperine were carried out in separate experiments, on different sets of male albino rats of the Wistar strain weighing 120-125 g. The animals were maintained for 10 days on 18% casein containing semi-synthetic diet (Vijayalakshmi & Chandrasekhara, 1981) with free access to food and water. They were housed individually in stainless steel metabolism cages, which permitted collection of urine and faeces. The animals weighed 150 - 160 g on the day of administration of spice principles. The oral dosages (mg / kg body weight) of the three spice principles were: Capsaicin: 30 mg; Piperine: 170 mg and Curcumin: 500 mg. These spice principles were administered by gavage to overnight fasted animals in the form of a suspension in 1.0 ml refined peanut oil. Different sets of the spice principle administered animals (6 animals per set) were sacrificed under ether anaesthesia at time intervals 1h, 3h, 6h, 1d, 2d, 4d and 8d following the oral administration. Blood, liver, kidney and intestine samples were collected. Urine and faeces were collected every 24 h for 8 days.

In a separate set of experiment, curcumin was orally administered (500 mg / kg body weight) concomitant with piperine (20 mg / kg body weight). In this case, groups of animals were sacrificed at time intervals: 3 h, 1d, 2d, 4d and 8d. Blood, liver, kidney

and brain tissues were collected. Urine and faeces were collected every 24 h for 8 days.

Blood samples were centrifuged at 3000 rpm to obtain serum. Faeces samples were dried at 80ºC, and then powdered. Urine samples were concentrated to a convenient smaller volume by flash evaporation. The serum and urine samples were extracted for the spice principle with ethyl acetate. Various other tissue samples and faeces samples were extracted for the spice principle by Folch procedure with chloroform-methanol (2:1 v/v) (Folch *et al*, 1957). The extracts were analyzed for the concerned spice principle by an appropriate HPLC procedure. Tissues from rats not dosed with any spice principle served as controls. Total blood volume (ml) in the rat was calculated using the formula: (Body weight in gram x 0.0778 = Blood volume).

Quantitation of spice principles by HPLC:

Quantitation of spice principles in the extracts cleaned up by passing through 2μ membrane filter was made by HPLC in a Shimadzu HPLC LC-10AT system consisting of a photo diode array detector, binary pump and manual sample injector. Chromatographic separation was accomplished using SGE 250 X 4.6 mm SS Excil C_{18} 10-μm column (Fig. 2). Assay performance and reproducibility were confirmed using internal standards. Curcumin was analyzed by isocratic elution at 425 nm. Sample (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing acetonitrile-water-acetic acid (50:49:1 v/v/v) at a flow rate of 1 ml/min (Asai & Miyazawa, 2000). Quantitation of curcumin was made from peak area ratio, which was based on a calibration curve generated from standard curcumin. Piperine analysis was performed on reverse phase column with an isocratic elution at 345 nm (Bajad *et al*. 2002). Ten μl piperine sample was eluted with a mobile phase containing a mixture

of 25 mM potassium dihydrogen phosphate (pH 4.5) and acetonitrile (35 : 65 v/v) at a flow rate of 1.0 ml/min. Quantitation of piperine was made from peak area ratio, which was based on a calibration curve generated from standard piperine. Capsaicin in the samples was separated and quantified by isocratic HPLC method using UV detection at a wavelength of 280 nm (Saria *et al*. 1981). An aliquot (10 μl) was injected on to a reverse phase column and eluted with a mobile phase containing a mixture of methanol-water (60:40 v/v) at a flow rate of 1.0 ml/min. Quantitation of capsaicin was made from peak area ratio, which was based on a calibration curve generated from standard capsaicin.

Results and Discussion

Tissue distribution and excretion of three spice principles – curcumin, capsaicin and piperine following their oral intake was examined in albino rats. These three bioactive compounds of spices share a considerable extent of structurally similarity. The oral dosages of the three spice principles examined in this study correspond to the respective effective dosages previously well documented to bring about the beneficial physiological effects. Our earlier study had indicated differences in the rate of absorption of these three spice principles in everted rat intestinal sacs. Among the three spice active principles examined for their absorption potential from the everted rat intestines, capsaicin is absorbed to a least extent for a given concentration of the spice principle. The absolute amounts of piperine absorbed in this *in vitro* system exceeded the amounts of curcumin. Only a minimum portion of the absorbed curcumin was traced in serosal fluid at the end of 3 h incubation period, while most of it was still present in intestinal tissue. The relatively lesser recovery of curcumin suggested transformation of the compound to a certain extent during absorption.

Tissue distribution and excretion of orally administered capsaicin:

The distribution of capsaicin in the various tissues namely, blood, liver, kidney and intestine is given in Table 7. Upon oral administration of capsaicin to rats at a dose of 30 mg / kg, 1.24 – 24.4 % of administered capsaicin was detected in blood, liver, kidney and intestine in 1 – 24 h. Whereas a maximum distribution of 24.36 % of administered capsaicin in these tissues was seen at 1 h, the same gradually reduced to 1.24 % in 24 h and to 0.057 % in 48 h. No capsaicin was detectable in these tissues beyond 96 h. Highest concentration of administered capsaicin in serum (1.90 µg/ml) was seen at 1h, which decreased to less than half (0.83 µg/ml) in 6 h and further to about 2.5 % (0.05 µg/ml) by 24 h. A small amount of capsaicin was still seen in blood at 48 h (0.006 µg/ml serum) but not thereafter. Highest concentration of capsaicin was seen in liver at 3 h; the decline in concentration of capsaicin in liver was fast thereafter. Its concentration in liver which was about 45 µg at 3 h was reduced to about 8.7 µg in 24 h. Absorption of orally administered capsaicin appears to be very rapid relative to the other two spice principles as indicated by its highest concentration in intestinal tissue at 1h. Capsaicin content of intestine decreased from 1057 µg in 1 h to 249 µg in 6 h and further to 43.5 µg by 24 h. No capsaicin was detectable in kidney beyond 2 day and in intestine or liver beyond 4 day.

About 6.3 % of the administered capsaicin was excreted as such in the faeces over a period of 4 days, with the peak excretion occurring on the first day oral intake (Table 8). Thus, nearly 94 % of orally administered capsaicin is absorbed. Only a small portion of capsaicin was also excreted intact in urine (0.095 %). Kawada *et al* (1984)

Table 7. Tissue distribution of orally administered capsaicin (30 mg/kg) in rat

Time (h)	Serum (µg/ml)	Blood (µg/ total blood)	Liver (µg/ whole tissue)	Kidney (µg/whole tissue)	Intestine (µg/whole tissue)
1	1.90 ± 0.18	11.11 ± 1.05	24.7 ± 2.1	3.61 ± 0.32	1057.0 ± 157.0
3	1.47 ± 0.09	8.59 ± 0.53	44.7 ± 3.37	5.71 ± 0.33	700.2 ± 42.2
6	0.83 ± 0.10	4.85 ± 0.59	14.8 ± 1.50	6.73 ± 0.45	249.3 ± 24.0
24	0.05 ± 0.01	0.29 ± 0.06	8.71 ± 2.55	3.35 ± 0.45	43.5 ± 3.75
48	0.006 ± 0.001	0.035 ± 0.006	0.60 ± 0.03	0.48 ± 0.09	1.14 ± 0.21
96	0.00	0.00	0.045 ± 0.005	0.00	0.72 ± 0.01
192	0.00	0.00	0.00	0.00	0.00

Values are mean ± SEM of 6 rats

4.5 mg Capsaicin was orally administered to rats weighing about 150 g.

Table 8. Elimination of orally administered capsaicin (30mg/kg) in rat

Day	Faeces	Urine
1	174.0 ± 11.3	4.05 ± 0.45
2	99.8 ± 5.03	0.225 ± 0.035
3	11.3 ± 1.25	0
4	0.375 ± 0.032	0
5	0	0
6	0	0
7	0	0
8	0	0
Total	285.5 (6.34% of administered dose)	4.275 (0.095% of administered dose)

Values expressed in µg are mean ± SEM of 6 rats

4.5 mg Capsaicin waas orally administered to rats weighing about 150 g.

have reported from an *in vivo* study in rats that gastrointestinal absorption of capsaicin is rapid and about 85% of the dose was absorbed within 3 h. The same authors have also evidenced that the absorbed capsaicin is readily transported to the portal blood (about 85 %) and partly metabolized during absorption to 8-methyl nonanoic acid. Donnerer *et al* (1990) report from a study conducted on anaesthetized rats that intragastrically administered capsaicin was readily absorbed from the GI tract; unchanged compound was present in portal blood, but was almost completely metabolized before reaching the general circulation after an effective metabolism in liver.

Tissue distribution and excretion of orally administered piperine:

The distribution of piperine in the various tissues namely, blood, liver, kidney and intestine is given in Table 9. Upon oral administration of piperine to rats at a dose of 170 mg / kg by gavage, 3.1 – 10.8 % of administered piperine was detected in blood, liver, kidney and intestine from 1 - 24 h. A maximum of 10.8 % of administered piperine was seen in these tissues by 6 h after administration. The amount of piperine in serum which was 6.07, 9.75, and 11.06 µg/ml at 1, 3 and 6 h respectively, declined drastically to 0.93 µg/ml at the end of 24 h and was nil in the blood after 4 d. In rats administered piperine only 0.15 - 0.39 % could be detected in the liver during first 24 h, with a maximum of 0.39 % present at 6 h. The concentration of piperine in kidney also was maximum at 6 h (0.37 %). Piperine concentration in the intestinal tissue gradually increased from 1.16 mg to a maximum of 1.96 mg by 6 h, and declined thereafter. The amount of piperine present in tissues significantly reduced by 48 h (0.30 %) and was not detectable beyond 96 h in blood, liver and intestine. The elimination pattern of piperine is presented in Table 10. Piperine was not detectable in urine at any time

Table 9. Tissue distribution of orally administered piperine (170 mg/kg) in rat

Time (h)	Serum (µg/ml)	Blood (µg/ total blood)	Liver (µg/ whole tissue)	Kidney (µg/whole tissue)	Intestine (µg/whole tissue)
1	6.07 ± 0.82	35.53 ± 4.82	56.12 ± 1.01	12.51 ± 1.95	1450.0 ± 137.2
3	9.75 ± 1.07	57.03 ± 6.28	73.75 ± 2.05	22.0 ± 3.53	1725.2 ± 150.2
6	11.06 ± 0.80	64.71± 4.68	96.62 ± 5.71	91.75 ± 6.51	2450.3 ± 162.0
24	0.93 ± 0.16	5.48 ± 0.73	37.87 ± 3.81	28.75 ± 3.35	700.1 ± 112.5
48	0.37 ± 0.05	2.19 ± 0.36	4.77 ± 0.88	4.62 ± 0.47	62.3 ± 1.08
96	0.078 ± 0.005	0.46 ± 0.03	0.33 ± 0.04	0.66 ± 0.13	4.47 ± 0.88
192	0	0	0	0.63 ± 0.11	0

Values are mean ± SEM of 6 rats

25 mg Piperine was orally administered to rats weighing about 150 g.

Table 10. Elimination of orally administered piperine (170 mg/kg) in rat

Day	Faeces
1	737.0 ± 80.3
2	150.6 ± 15.8
3	20.0 ± 2.33
4	1.85 ± 0.35
5	0
6	0
7	0
8	0
Total	909.5 (3.64 % of administered dose)

Values expressed in µg are mean ± SEM of 6 rats

25 mg Piperine was orally administered to rats weighing about 150 g.

Piperine was not found in urine

interval. On the other hand, 3.64 % of the administered piperine was excreted as such in the faeces over a period of 4 days, with the peak excretion occurring on the first day of oral intake. Thus, absorption of the administered piperine was about 96%.

An earlier study by Bhat and Chandrasekhara (1986) on the tissue distribution of orally administered piperine in rats has reported a similar maximum concentration of piperine at 6 h in blood, liver, kidney and spleen. These authors however did not detect piperine in any of the tissue samples beyond 24 h, nor did they detect piperine in blood samples of 1 h and 24 h. Our present observation differs from this earlier report in that piperine could be detected in blood and other tissues even up to 4 days. The fact that employment of TLC - densitometry for the detection and estimation of piperine in tissue samples in this previous study, while HPLC procedures were used in the current investigation explains the observed deviation in the tissue distribution data.

Increased excretion of conjugated glucuronides, sulfates and phenols in the urine for up to 8 days following oral administration of piperine in rats has been earlier evidenced which are presumably the transformed products of piperine (Bhat & Chandrasekhara, 1986). It is suggested that piperine undergoes demethylenation of methylenedioxy group; glucuronidation and sulfation appear to be the major reactions in the metabolism of piperine in rats. These authors also have not observed excretion of piperine as such in urine of rats. These authors also infer from their data that most of the administered piperine is absorbed without any transformation during absorption by the intestine, and is later metabolized rapidly by other tissues.

Tissue distribution and excretion of orally administered curcumin:

The distribution of curcumin in the various tissues namely, blood, liver, kidney and intestine is given in Table 11. Upon oral administration of curcumin to rats at a dose of 500 mg / kg, 3.88 – 48.3 % of administered curcumin was detected in blood, liver, kidney and intestine from 1 - 24 h. Curcumin concentration was maximum in the intestine at the end of 1st h; the concentration of curcumin in the intestinal tissue gradually decreased from 36.2 mg at 1 h to 2.21 mg by 24 h. No curcumin was detectable in the intestine after 4 days. Concentration of curcumin in blood reached maximum (83.8 µg/ml) at 6th h and continued to remain at significantly higher level (52.6 µg/ml) even at 24 h. Curcumin concentration was maximum in liver and kidney at 6 h. Whereas curcumin was not detectable in kidney after 24 h, the same was present in liver tissue even up to 4 days.

Vijayalakshmi and Chandrasekhara (1980), who have studied tissue distribution of orally administered curcumin in rats, could not detect intact curcumin in blood, liver and kidney up to 24 h. Hence these authors have inferred that curcumin probably undergoes transformation as it is being absorbed from the intestine. Our observation of the presence of intact curcumin in blood and other tissues in the current study differs from this earlier report. This could be explained by the more dependable HPLC method employed by us for the detection and analysis of curcumin.

The elimination pattern of orally administered curcumin is presented in Table 6. Unchanged curcumin was excreted mostly in the faeces to an extent of 36.5 % of the single oral administration over a period of 8 days, tapering off especially after 5 days. Maximum faecal excretion of curcumin occurred during first 24 h and gradually declined thereafter. Nearly 34% of the eliminated curcumin was seen in the faeces of

Table 11. Tissue distribution of orally administered curcumin (500mg/kg) in rat

Time (h)	Blood (µg/ml)	Blood (µg/ total blood)	Liver (µg/ whole tissue)	Kidney (µg/Whole tissue)	Intestine (mg/whole tissue)
1	2.94 ± 0.21	34.40 ± 2.46	16.46 ± 1.49	1.73 ± 0.33	36.19 ± 3.10
3	34.69 ± 2.21	405.9 ± 25.9	32.29 ± 4.25	4.39 ± 0.79	17.79 ± 1.68
6	83.80 ± 5.46	980.5 ± 63.9	135.2 ± 5.26	9.03 ± 1.11	11.83 ± 0.83
24	52.56 ± 2.54	615.0 ± 29.7	86.46 ± 1.90	1.98 ± 0.76	2.21 ± 0.43
48	9.57 ± 1.02	112.0 ± 11.9	20.00 ± 2.31	0	0.255 ± 0.025
96	0.73 ± 0.08	8.54 ± 0.94	4.77 ± 0.69	0	0.027 ± 0.006
192	0	0	0	0	0

Values are mean ± SEM of 6 rats

75 mg Curcumin was orally administered to rats weighing about 150 g.

Table 12. Elimination of orally administered curcumin (500mg/kg) in rat

Day	Faeces	Urine
1	9.42 ± 0.66	0.105 ± 0.005
2	7.06 ± 0.46	0.019 ± 0.001
3	5.19 ± 0.43	0.006 ± 0.001
4	2.85 ± 0.48	0
5	1.75 ± 0.28	0
6	0.75 ± 0.10	0
7	0.24 ± 0.03	0
8	0.12 ± 0.02	0
Total	27.38 (36.5% of administered dose)	0.13 (0.173% of administered dose)

Values expressed in mg are mean ± SEM of 6 rats

75 mg Curcumin was orally administered to rats weighing about 150 g.

first 24 h. Excretion of intact curcumin in the urine was only minimal and accounted for 0.173 % of the administered dose. Thus, about 63.5 % of the curcumin dose was absorbed. This figure for the absorption of curcumin is more than twice that reported by Wahlstrom and Blennow (1978) and is about the same as what was observed by Vijayalakshmi and Chandrasekhara (1980) by administering 80 and 400 mg curcumin / rat. The reason for the higher absorption in the present investigation could be the lower dose (500 mg / kg body wt) used. These authors have not detected curcumin in the urine. These earlier authors have reported elevated excretion of conjugated glucuronides and sulfates in the urine of curcumin administered animals. The glucuronide excretion returned to normal by 7^{th} day after curcumin administration, while higher conjugated sulfate excretion continued up to 6 weeks.

Tissue distribution and excretion of curcumin orally administered concomitant with piperine:

The medicinal properties of curcumin are likely to be under-utilised because of poor bioavailability due to its rapid metabolism in the liver and intestinal wall. Piperine, the active principle of black pepper is known to be a strong inhibitor of hepatic and intestinal aryl hydrocarbon hydroxylation and glucuronidation. Piperine has been documented to enhance the bioavailability of a number of therapeutic drugs as well as phytochemicals by this very property (Srinivasan, 2006). In this context, tissue distribution and excretion of curcumin orally administered concomitant with piperine was evaluated in rats.

The distribution of curcumin in blood, liver, and kidney when orally administered (500 mg/kg) concomitant with piperine (20 mg/kg) is presented in Table 13. Upon oral administration of curcumin to rats along with piperine, curcumin could be found in blood

Table 13. Tissue distribution of curcumin (500mg/kg) in rat after oral administration concomitant with piperine (20 mg/kg).

Time (h)	Blood (µg/ml)	Blood (µg/ total blood)	Liver (µg/ whole tissue)	Kidney (µg/whole tissue)	Brain (µg /whole tissue)
3	42.05 ± 3.77	492.0 ± 44.1	52.07 ± 2.24	5.96 ± 1.14	ND
24	64.29 ± 1.69	752.2 ± 19.8	134.3 ± 6.94	2.98 ± 1.03	1.84 ± 0.33
48	8.04 ± 1.09	94.07 ± 12.75	43.09 ± 1.53	1.16 ± 0.37	5.87 ± 0.38
96	2.53 ± 0.40	29.60 ± 4.68	13.00 ± 2.46	0.48 ± 0.10	0.43 ± 0.16
192	0.64 ± 0.08	7.49 ± 0.94	1.86 ± 0.20	0.00	ND

Values are mean ± SEM of 6 rats

75 mg Curcumin was orally administered concomitant with 3 mg Piperine to rats weighing about 150 g.

ND : Not determined

Table 14. Elimination of curcumin (500mg/kg) in rat following oral administration concomitant with piperine (20 mg / kg)

Day	Faeces	Urine
1	7.13 ± 0.98	0.63 ± 0.09
2	4.51 ± 0.57	0.25 ± 0.03
3	2.78 ± 0.40	0.103 ± 0.007
4	1.10 ± 0.24	0.075 ± 0.005
5	0.49 ± 0.08	0.019 ± 0.002
6	0.37 ± 0.06	0
7	0.15 ± 0.02	0
8	0.07 ± 0.01	0
Total	16.60 (22.13 % of administered dose)	1.077 (1.44 % of administered dose)

Values expressed in mg are mean ± SEM of 6 rats

75 mg Curcumin was orally administered concomitant with 3 mg Piperine to rats weighing about 150 g.

even up to 8 days. For a similar concentration of administered curcumin, the concentration of intact curcumin present in blood at 3 h, 24 h, 96 h and 192 h was definitely higher than what is found when curcumin was administered alone. At 3 h and 24 h, the amount of curcumin in blood was about 20 % higher than that found when curcumin was administered alone. Similarly, the amounts of curcumin present in liver tissue at 3 h, 24 h, 48 h, 96 h and 192 h was much higher than what is found when curcumin was administered alone. Curcumin concentrations in liver at 3h, 24 h, 48 h and 96 h were respectively 61, 55, 115 and 173 % higher that what is found when curcumin was administered alone. Curcumin concentration in kidney found at 3 h and 24 h was higher than what is seen when curcumin was administered alone; curcumin could be detected in kidney even at 2 and 4 day intervals. Thus, curcumin stayed significantly longer in the body tissues when administered concomitant with piperine. Curcumin was also detected in the brain tissue at 24, 48 and 96 h with a maximum at 48 h. The amount of curcumin present in brain at 48 h exceeded the amount present in kidney (5.87 µg vs. 1.16 µg).

The elimination pattern of curcumin orally administered concomitant with piperine is presented in Table 8. Unchanged curcumin was excreted mostly in the faeces to an extent of 22.1 % of the single oral administration over a period of 8 days. Maximum faecal excretion of curcumin occurred during first 24 h and gradually declined thereafter. Nearly 43 % of the eliminated curcumin was seen in the faeces of first 24 h. Excretion of intact curcumin in the urine was relatively higher compared to what was seen when curcumin was administered alone and accounted for 1.43 % of the curcumin dose administered. Further, excretion of intact curcumin in the urine continued up to 5^{th} day in this case. Thus, about 78 % of the oral curcumin dose was

absorbed when administered concomitant with piperine. The extent of curcumin absorption was thus significantly higher than what is found when similar dose was administered alone.

Shoba *et al* (1998) have studied the effect of combining piperine, on the bioavailability of curcumin in rats and in human volunteers. When curcumin was given at a dose 2 g/kg to rats, moderate serum concentrations were achieved over a period of 4 h. Concomitant administration of piperine 20 mg/kg increased the serum concentration of curcumin for a short period of 1-2 h post drug. Time to maximum was significantly increased while elimination half life and clearance significantly decreased, and the bioavailability was increased by 154 %. On the other hand in humans after a dose of 2 g curcumin alone, serum levels were either undetectable or very low. Concomitant administration of piperine 20 mg/kg produced much higher concentrations from 0.25 to 1 h post drug; the increase in bioavailability was 2000%. The authors infer from their study that in the dosages used, piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects. Thus, the results of the present investigation which has evidenced prolonged occurrence of curcumin in tissues when consumed concomitant with piperine, concurs with the earlier report of Shoba *et al* (1998). The extent of absorption of curcumin when administered concomitant with piperine is 78 % compared to 63.5 % absorption observed when curcumin was administered alone. Thus, piperine has not only enhanced the bioavailability of curcumin so that it remains in the body tissues longer by reducing the rate of its metabolic breakdown, but also by enhancing the extent of intestinal absorption. Significantly higher excretion of unmetabolized

curcumin in the urine when administered in presence of piperine is an additional evidence to this inference.

Epidemiological studies suggest that anti-inflammatory drugs reduce the risk of Alzheimer's disease (Hoozemans *et al*. 2003). There is also substantial *in vitro* data indicating that curcumin has antioxidant, anti-inflammatory, and anti-amyloid activity (Ringman *et al*. 2005). In addition, studies in animal models of Alzheimer's disease indicate a direct effect of curcumin in decreasing the amyloid pathology of Alzheimer's disease. In this context, it is of interest to know if curcumin reaches the brain tissue crossing the blood brain barrier. The distribution of curcumin in brain tissue following its oral intake and its persistence in this tissue for a considerable duration is evidenced in the present investigation. This is the first report of the presence of curcumin in the brain tissue following its administration.

The numerous biological effects attributable to the spice principles – curcumin, capsaicin and piperine indicate that these compounds could serve as functional food ingredients. Development of drugs out of such natural constituents requires information on pharmacokinetic and safety aspects. Animal studies have indicated that even at much higher dietary levels (up to 100 times the normal intake among Indian population), turmeric, red pepper and black pepper have no adverse effects on growth, organ weights, nitrogen balance and blood constituents (Srinivasan, 2005). The pharmacological safety of consumption of curcumin has been established up to 100 mg / day in humans and up to 5 mg / day in rats (Commandeur & Vermeulen, 1996). The pharmacokinetic properties of intraperetoneally administered curcumin have investigated in mice (Pan *et al*, 1999). Ireson *et al* (2001) suggest that the bioavailability of curcumin is greatest in the colon based on their observation. Because

GI tract seems to be exposed more prominently to unmetabolized curcumin than any other tissue, the compound has the potential as a colorectal cancer chemopreventive agent. Human trials have evidenced that regular oral intake of 3.6 g curcumin / day results in levels of curcumin in the GI tract that elicit pharmacodynamic changes, such that it can serve as a colorectal cancer chemopreventive agent (Garcea *et al*, 2005). Absence of quantifiable curcumin in the plasma has been observed even after a dose of 3.6 g of curcumin (Garcea *et al*, 2005) and a oral dose of 30 -180 mg curcumin (Sharma *et al*, 2001). Doses of 4-12 g are shown to yield curcumin levels of only < 2 μmol/L within 1 h of administration (Cheng *et al*, 2001).

Conclusions

In summary, a study of the tissue distribution and elimination of the three structurally similar bioactive spice principles - curcumin, capsaicin and piperine following their oral intake in rats indicated that intestinal absorption of capsaicin was relatively rapid. Absorption of the orally administered dose of capsaicin, piperine and curcumin was 94, 96 and 63.5% respectively. Concentration of capsaicin and piperine in blood was maximum at 1h and 6h respectively, and decreased markedly thereafter. Curcumin concentration was maximum in blood at 6 h and remained at significantly higher level even at 24 h. Maximum tissue distribution of 24.4 % of administered capsaicin was seen at 1 h, while the same was not detectable after 4 days. Only a small portion of the administered dose of capsaicin (< 0.1%) and curcumin (0.173 %) was excreted in urine, whereas piperine was not detectable in urine. Enhanced bioavailability of curcumin was evidenced when the same was orally administered concomitant with piperine (20 mg/kg). Intestinal absorption of curcumin was relatively

higher in this case, and it stayed significantly longer in the body tissues. Curcumin was detected in brain at 24, 48 and 96 h with a maximum at 48 h.

CHAPTER - IV

INTERACTION OF SPICE PRINCIPLES WITH SERUM ALBUMIN

Section - A: Binding of piperine with human serum albumin: A spectrofluorimetric study

Section - B: Studies on the interaction of capsaicin with human serum albumin

INTERACTION OF SPICE PRINCIPLES WITH SERUM ALBUMIN

Introduction

The health beneficial physiological effects of the three common spices – turmeric (*Curcuma longa*), red pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) are incidentally attributable to their respective active principles - curcumin, capsaicin and piperine (Srinivasan, 2005). Among a wide range of health-beneficial physiological effects curcumin is documented to exert are its antioxidant, anti-inflammatory and anti-carcinogenic potential. Capsaicin is endowed with several health beneficial attributes which include hypolipidemic property (Srinivasan *et al*, 2004), antilithogenic property (Hussain & Chandrasekhara, 1993 & 1994), antioxidant property (Reddy & Lokesh, 1992) and anti-inflammatory influence (Reddy & Lokesh, 1994a; Joe & Lokesh, 1997). Capsaicin has been shown to be useful in diabetic neuropathy (The Capsaicin study group, 1992).

Piperine has been endowed with several health beneficial physiological and pharmacological properties. Among these, the most important is its ability to enhance bioavailability of therapeutic drugs and phyto-nutrients (Shoba *et al*, 1998; Velpandian *et al*, 2001). Piperine enhances the bioavailability of these concomitantly administered compounds by virtue of inhibiting the drug metabolizing enzymes, thus retarding their clearance from the body (Koul *et al*, 2000). The most far-reaching attribute of piperine has been its inhibitory influence on enzymatic drug bio-transforming reactions in liver. It strongly inhibits hepatic and intestinal aryl hydrocarbon hydroxylase and UDP-glucuronyl transferase (Atal *et al*, 1985). It is also evident that enhancement of

bioavailability of these compounds by piperine is partly attributable to an increased absorption, facilitated by alterations in the intestinal epithelial membrane lipid dynamics and permeation characteristics. This is associated with induction in the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier (Khajuria *et al*, 2002). Piperine has been demonstrated in *in vitro* studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Mittal & Gupta, 2000). Piperine treatment has also been evidenced to lower lipid peroxidation *in vivo* and beneficially influences cellular thiol status, antioxidant molecules and antioxidant enzymes in a number of experimental situations of oxidative stress (Vijayakumar *et al*, 2004). Piperine, while it is non-genotoxic has in fact been found to possess antimutagenic and antitumour influences (Srinivasan, 2006).

These three spice principles share a considerable amount of structural homology. A number of animal studies indicate that these compounds reach the target tissues to exhibit their diverse physiological activities. While all these three spice principles are essentially lipophilic, it would be most relevant to understand how these compounds are transported in blood circulation to various tissues in the body following their intestinal absorption. The mode of transport of spice active compounds *in vivo* is not clear, except for few studies in the case of curcumin. Many lipophilic molecules like long-chain fatty acids are known to be carried by albumin in blood (Spector, 1975).

Human serum albumin (HSA), a heart-shaped protein with 585 amino acids is organized in to three homologous domains (I, II and III), each domain consisting of two

sub-domains (A & B) that share common structural elements (He & Carter, 1992). A total of 17 disulfide bridges, which are exclusively present in intra-domain and conserved across species contribute towards its impressive thermo-stability. The importance of serum albumin in pharmacology derives from its high concentration in blood (0.6 mM in plasma) and from its ability to bind to an extraordinary diverse range of ligands such as drugs, fatty acids, metabolites and phytonutrients. Serum albumin increases the solubility of hydrophobic ligands in plasma and modulates their delivery to cells. The crystal structure studies of the binding sites of serum albumin and extensive characterization of the drug binding to HSA by spectroscopic techniques have revealed that binding site I is dominated by the strong hydrophobic interactions with most neutral, bulky, heterocyclic compounds. On the other hand, binding site II involves ion-dipole, dipole-dipole, Van der Walls, hydrogen bonding interactions with carboxylic acids (Sudlow *et al*, 1975; Yamasaki *et al*, 1994; Moreno *et al*, 1999). Thermodynamically, the recognition of the ligand by HSA at site I is an entropy driven reaction with positive or minimal negative entropic contributions. At site II, it is mainly an enthalpy driven complexation with large negative entropic gains (Urien *et al*, 1994; Aki *et al*, 1994).

Interaction of curcumin, the bioactive principle of the spice - turmeric (*Curcuma longa*) with HSA has been examined by spectroscopic studies (Reddy *et al*, 1999; Zsila *et al*, 2003). This compound was found to interact with HSA at two binding sites, one with high affinity and the other with low affinity. Curcumin binding did not change the conformation of HSA. Even though piperine and capsaicin have been understood to exhibit many physiological and pharmacological effects, their interaction with mammalian biopolymers such as HSA is almost unexplored. Only one recent study

(Zsila *et al*, 2005) reveals that piperine associates with β-lacto globulin non-covalently as shown by extrinsic CD bands induced by the interaction of the ligand chromophore with the protein. The affinity between ligand and HSA plays a crucial role in determining the bioavailability of many bioactive compounds such as drugs, phytonutrients, etc. In the present investigation, interaction of the spice principles – piperine and capsaicin with HSA has been examined employing spectroscopic measurements – fluorescence quenching and circular dichroism. Such a study assumes importance in the context of understanding the carrier role of serum albumin for piperine and capsaicin transport in blood under physiological conditions.

Section - A: Binding of piperine with human serum albumin: A critical spectrofluorimetric study

Summary

In the present investigation, interaction of the spice principle – piperine with HSA has been examined employing spectroscopic measurements – fluorescence and circular dichroism. Piperine, the bioactive alkaloid compound of the spice - black pepper exhibits a wide range of beneficial physiological and pharmacological activities. Being essentially water-insoluble, piperine is presumed to be assisted by serum albumin for its transport in blood. In this study, the binding of piperine to serum albumin was examined by employing steady state and time resolved fluorescence techniques. Binding constant for the interaction of piperine with human serum albumin was observed to be 0.5 X 10^5 M^{-1} having stoichiometry of 1:1. The binding constant was invariant with temperature in the range of 17^0C to 47^0C. At 27^0C, the van't Hoff enthalpy ΔH° was zero. ΔS° and ΔG° were found to be 21.4 $cal.mol^{-1}K^{-1}$ and – 6.42 $kcal.mol^{-1}$. The binding constant increased with the increase of ionic strength from 0.1 M to 1.0 M of sodium chloride. The decrease of Stern-Volmer constant with increase of temperature suggested that the fluorescence quenching is static in nature. Piperine fluorescence showed a blue shift upon binding to serum albumin, which reverted with the addition of ligands such as triode benzoic acid and hemin. The distance between piperine and tryptophan after binding was found to be 2.79 nm by FRET calculations. The time resolved fluorescence study with BSA revealed that the amplitude of longer lifetime component decreased significantly with the increase in concentration of piperine. The steady state and time resolved fluorescence measurements suggest the binding of piperine to the sub-domain IB of serum albumin. These observations are

significant in understanding of the transport of piperine in blood under physiological conditions.

Materials and methods

Materials

Human serum albumin (HSA), Bovine serum albumin (BSA), warfarin, diazepam, triiodobenzoic acid, hemin and Trizma base were from M/s Sigma Aldrich Chemical Co. (St. Louis, USA). Piperine and 8-anilino-1-naphthalene sulfonic acid (ANS) were from M/s Aldrich Chemical Co., Milwaukee, USA. All other chemicals used here were of analytical grade.

Purification of human and bovine serum albumin

The higher molecular weight aggregates associated with commercial preparations of human and bovine serum albumin were removed by size exclusion chromatography on a Sephadex G-100 column (120 X 1 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Fractions of 1 ml were collected at a flow rate of 10 ml/ h and the purity was ascertained by SDS-PAGE (Laemmli, 1970). Protein concentration of the HSA fractions was determined using a value of 5.30 for $E_{1\%}$ at 278 nm (Clark *et al*, 1962). BSA concentration was estimated using a value of 6.67 for $E_{1\%}$ at 279 nm (Föster & Sterman, 1956).

Absorbance measurements

All the absorbance measurements were made at 27°C using a 1601 Shimadzu double beam UV spectrophotometer in a 1 cm path length cell.

Fluorescence measurements

Shimadzu RF 5000 spectrofluorophotometer attached with a thermostated circulating water bath was employed for fluorescence measurements. The spectrofluorophotometer was calibrated for wavelength accuracy and S/N ratio as suggested by the manufacturer. The solution in the cuvette was subjected stirring with the help of Hellma cuv-o-stir®. 10 mm path length cuvette with sample in 0.05M Tris-HCl buffer (pH 7.4) was used for measurements.

Serum albumin samples were centrifuged at 26,000 x g for 30 min to remove any aggregates. Stock solution (1.25 mM) of piperine in methanol was added in increments of 2 μl to 1 μM HSA in 0.05 M Tris-HCl, pH 7.4. Fluorescence quenching of HSA by piperine was followed at 27 ± 0.2°C. The excitation and emission wavelengths were set at 280 nm and 333 nm, respectively. Slit widths for excitation and emission were 5 and 10 nm, respectively. Blank titrations, with methanol, were carried out to correct the quenching due to methanol. The fluorescence readings were corrected for inner filter effects due to absorption of light by the addition of ligand to the protein-ligand complex (Ward, 1985). Stoichiometry was determined by plotting log F_o – F/ F – F_∞ versus log [μmole piperine] as described by Chipman *et al* (1967). Where F_o, F and F_∞ are the fluorescence intensities at zero, finite and infinite concentrations of the quencher. Quenching, as a function of piperine concentration, was analyzed in terms of binding of the piperine by HSA using established procedures (Rao & Cann, 1981). Thus, if it is assumed that the binding of each piperine molecule causes the same degree of quenching and that binding is statistical, the intrinsic piperine – binding constant, *K*, is given by the equation,

$$K = \beta/(1-\beta) \,.\, 1/C_f \quad \ldots\ldots \text{Equation I}$$

where β = Q / Q_{max} and C_f = C_T - $n\beta T$, in which Q is the corrected percentage quenching; Q_{max}, the maximal quenching; C_f, the molar equilibrium concentration of unbound piperine; C_T, the molar constituent concentration of piperine; T, the molar constituent concentration of serum albumin; and n is the binding stoichiometry (Ward, 1985). The value of K is given by the slope of a plot of β / 1-β against C_f. Q_{max} has been determined by extrapolation of a double reciprocal plot of 1/ Q vs. 1/C, to 1/C = 0. In both cases, the data are fitted to a straight line by the method of least squares.

Determination of Stern-Volmer constant

The Stern-Volmer constant was calculated by fluorescence quenching of HSA by piperine, in the range of 17 - 47°C. Data was analyzed using the modified Stern-Volmer equation (Lakowicz, 1999).

$$\frac{F_0}{\Delta F} = \frac{1}{fK\,[Q]} + \frac{1}{f}$$ …….. Equation II

where F_o is the fluorescence in the absence of quencher, ΔF is the difference in fluorescence in the absence and presence of the quencher at concentration [Q], K is the Stern-Volmer quenching constant, and f is the fraction of initial fluorescence which is accessible to the quencher. The plots of F_o/ ΔF versus 1/[Q] yields f^{-1} as the intercept, and $(fK)^{-1}$ as the slope.

Effect of temperature

The effect of temperature in the range 17 - 47°C on the binding constant of piperine with human serum albumin was determined by fluorescence quenching studies using a

Shimadzu RF 5000 spectrofluorophotometer and appropriate blanks. The concentrations of HSA and piperine were same as given above.

Effect of ionic strength

The effect of ionic strength on the binding constant of piperine with HSA was determined by increasing concentrations of sodium chloride (0 to 1M) in Tris-HCl buffer by fluorescence titration at 27 $\pm$ 0.2°C as given above. HSA and piperine were used in the same concentrations as in the quenching studies and using appropriate blanks.

Fluorescence measurements of piperine

Fluorescence properties of piperine were followed by excitation at 345 nm and emission range of 350 to 550 nm. Excitation and emission slit widths were set at 10 nm each. A stock solution of HSA (30 mg/ml) was added in increments of 10 μl to 1 % methanolic solution of 10 μM piperine in 0.05 M Tris-HCl, pH 7.4.

Competitive ligand binding studies

A 10 μM 1:1 complex of HSA and piperine was titrated against warfarin, diazepam, triiodo benzoic acid (TIB) and hemin. Stock solutions (10mM) of warfarin, diazepam and triiodo benzoic acid were prepared in dimethyl sulfoxide. A stock solution of hemin (10 mM) was prepared in 10mM sodium hydroxide. These ligand stock solutions were added to the piperine – HSA complex solution such that the final concentration of the marker ligand was 12.5 μM. Fluorescence properties of piperine were monitored after excitation at 345 nm in the emission range of 350 to 550 nm. Excitation and emission slit widths were set at 10 nm each. Fluorescence of warfarin did not interfere in the piperine's emission region.

Effect of ANS on binding of piperine

HSA (1μM) was saturated with ANS (3 μM) in 50 mM Tris-HCl (pH 7.4) and 5 μl increments of 1μM methanolic solution of piperine added to this solution. Concentration of ANS was determined by its molar absorption coefficient of 4.95 x 10^3, at 350 nm (Weber & Young, 1964). The decrease in fluorescence of ANS bound HSA was recorded. Blank titrations with methanol were carried out and corrected for dilution. The excitation and emission wavelengths for ANS bound HSA were set at 375 and 467 nm respectively.

Fluorescence Anisotropy Measurements with warfarin

Fluorescence anisotropy measurements were recorded at 27 ± 0.2°C on a Shimadzu RF 5000 spectrofluorophotometer attached with UV polarizers (POLACOAT Co., USA). The temperature was maintained using a circulating water bath. Warfarin (5μM) and HSA (10μM) complex was titrated with 2μl increments of piperine (20mM) dissolved in methanol. The data were recorded by setting the excitation and emission wavelengths at 310 and 385nm, respectively. The excitation and emission slit widths were 5 and 10 nm, respectively.

For anisotropy measurements, intensities of horizontal and vertical components of the emitted light ($I_{||}$ and $I_{\perp}$) were corrected for the contribution of scattered light. G, the grating factor that corrects for wavelength dependent distortions of the polarizing system was obtained using

$$G = F_{hv} / F_{hh} \quad \text{and} \quad I_{||} / I_{\perp} = (F_{vv})/ (F_{vh}) \ (F_{hh}/ F_{hv}), \qquad \text{.....} \quad \text{Equation III}$$

where F_{vv} , F_{vh}, F_{hv} and F_{hh} are the fluorescence intensity components, in which the subscripts refer to the horizontal (h) or vertical (v) positions of the excitation and emission polarizers separately. Anisotropy was calculated using the equation

$$A = (I_{||} / I_{\perp}) - 1/ (I_{||} / I_{\perp}) + 2 \quad \text{..... Equation IV}$$

Binding distance

There is a good overlap between the fluorescence emission spectrum of free HSA and UV-absorption spectrum of piperine. The efficiency of energy transfer as well as distance between piperine and tryptophan in serum albumin in the binding pocket was measured according to the Förster non-radiation energy transfer theory (Förster, 1967). The non-radiation energy transfer would occur between the donor and the acceptor of the fluorescence energy because of the proper overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor. The energy transfer efficiency E is related to the distance (r_0) between acceptor and donor, and also to the critical energy transfer distance (R_0), by the equation,

$$E = R_0^6/(R_0^6 + r_0)^6$$

Where R_0 is a characteristic distance, called the Förster distance or critical distance, at which the efficiency of transfer is 50%, computed from the relation,

$$R_0^6 = 8.8 \times 10^{-25} \kappa^2 N^{-4} \Phi$$

where κ^2 is the spatial orientation factor describing the relative orientation in space of the transition dipoles of the donor and acceptor, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor in the absence of the acceptor. Employing a water solution of tryptophan as reference (Φ = 0.14) (Wu & Brand, 1984), we determined the fluorescence quantum yield of HSA tryptophan to be

0.11. The refractive index N of the medium is the average value of water and organic solute (1.45) and K^2 is 2/3 for random orientation (Berde *et al*, 1979) and J is the overlap integral between the donor fluorescence emission spectrum and the acceptor absorption spectrum. J is given by,

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda / \sum F(\lambda)\Delta\lambda,$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ and its unit is cm^3 L mol^{-1}. Then the energy transfer efficiency E is,

$$E = 1 - F/F_0,$$

where F_0 = Fluorescence intensity of HSA alone and F = Fluorescence intensity of HSA with ligand. As the fluorescence emission of protein was affected by the excitation light around 280 nm, the spectrum ranging from 300 to 450 nm was chosen to calculate the overlapping integral.

Time resolved fluorescence studies with BSA

Time resolved fluorescence studies were performed on fluorescence lifetime spectrometer model 5000U; IBH, UK provided with a time correlated single photon counting set up. The second harmonic output from Tsunami mode locked picosecond laser was used as the exciting source. The mode locked 375 nm laser was focused on the sample and fluorescence photons from the sample were collected at the magic angle (54.7°) to avoid the distortions due to the rotational polarization. The emitted photons were detected by micro channel photomultiplier tube (Hamamatsu R3809U) after passing through the monochromator. To get the fluorescence decay curves, 10^4 counts at the peak were collected.

The fluorescence kinetic parameters were obtained by deconvoluting the excitation and instrument response function from the measured fluorescence decay. The data analysis was carried out by the software provided by IBH (DAS-6) which is based on reconvolution technique (equation - II) using iterative nonlinear least square methods.

$$I(t) = \sum_{i=1}^{3} \alpha_i \exp(-t/\tau_i), \sum_{\alpha_i} = 1 \qquad \text{…….. Equation V}$$

Where α_i is the amplitude and τ_i is the fluorescence life time of the *i*-th component. The goodness of the fit was determined from the randomness of the weighted residual distribution and χ^2. Where χ^2 is a statistical constraint, which is a measure of discrepancies between observed and expected frequencies in a time resolved fluorescence measurement.

Results

Steady state fluorescence measurements

The binding of HSA with Piperine has been monitored by following the quenching of relative fluorescence intensity of serum albumin, and is presented in Fig.1A. Quenching of fluorescence by piperine did not change the emission maxima. A maximum quench of 30% was observed at 10μM concentration of piperine. The same was also deduced form the double reciprocal plot of % Q vs. piperine concentration (Fig.1B). The stoichiometry of HSA-piperine complex has been estimated to be 1 : 1 ± 0.2 (Fig 3). The mass action plot presented in Fig. 2 has been constructed (using the value of n =1, extent of reaction reckoned from Fig.2). The binding constant given by the slope of this plot is 5.0 ± 0.2 X 10^4 M^{-1}. The Stern-Volmer constant decreases with increase in temperature, a characteristic that coincides with the static type of

quenching mechanism (Table-1). Static quenching arises from the formation of a dark complex between the fluorophore and the quencher.

Binding energetics

Effect of temperature on the interaction of piperine with serum albumin has been followed in the range of 17 to 47°C. The binding constant is invariant with temperature (Fig.4). Thus van't Hoff enthalpy $\Delta H°$ is zero. The binding reaction is entropy driven. $\Delta S°$ has been determined as 21.40 $cal.mol^{-1}K^{-1}$and $\Delta G°$ is found to be –6.42 $kcal.mol^{-1}$ at 27°C.

Effect of ionic strength on the binding of piperine to HSA

The effect of ionic strength on the HSA-piperine interaction was studied by varying the ionic strength of the buffer by the addition of sodium chloride (0.1 – 1M). It was observed that Q_{max} remained unaltered on increasing the ionic strength of the buffer implying no change in the binding geometry. The binding constant increased with increasing ionic strength (Fig. 5).

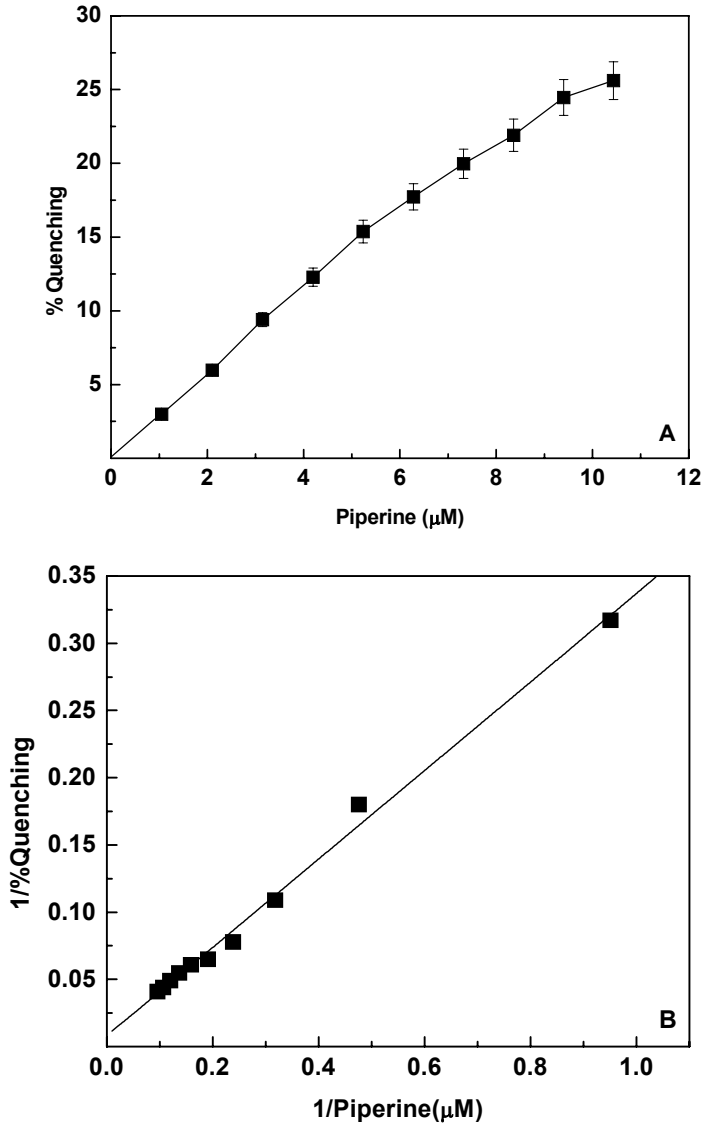


Fig. 1 *Quantitation of the interaction of HSA with piperine by fluorescence quenching.* HSA (1μM) in 50 mM Tris-HCl buffer, pH 7.4 was titrated with increasing aliquots of stock piperine solution (2 μl equivalent to 1 μM piperine per aliquot) in methanol and the % quench was recorded. Fluorescence quench was corrected due to absorption of light by piperine as mentioned in the text. The results are average of five sets of independent experiments.

A: Percent quench of fluorescence intensity as a function of constituent piperine concentration.

B: Double-reciprocal plot of data in A; Q_{max} = 28 ± 3 (± indicates probable error).

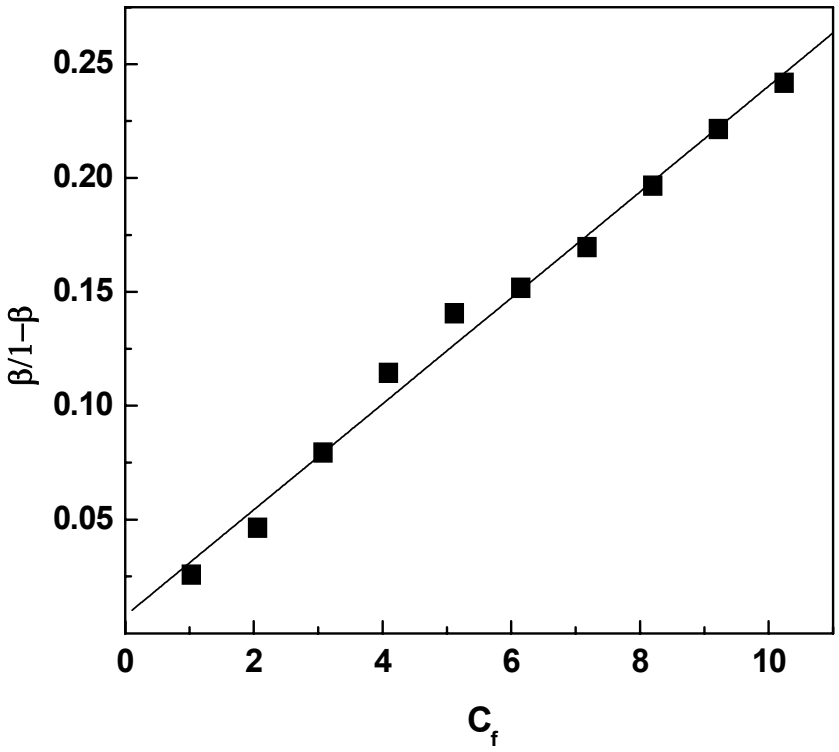


Fig. 2 *Mass action plot of quantitation of the interaction of HSA with piperine by fluorescence quenching*.

HSA (1μM) in 50 mM Tris-HCl buffer, pH 7.4 was titrated with increasing aliquots of stock piperine solution (2 μl equivalent to 1 μM piperine per aliquot) in methanol and the % quench was recorded. Fluorescence quench was corrected due to absorption of light by piperine as mentioned in the text. The results are average of five sets of independent experiments.

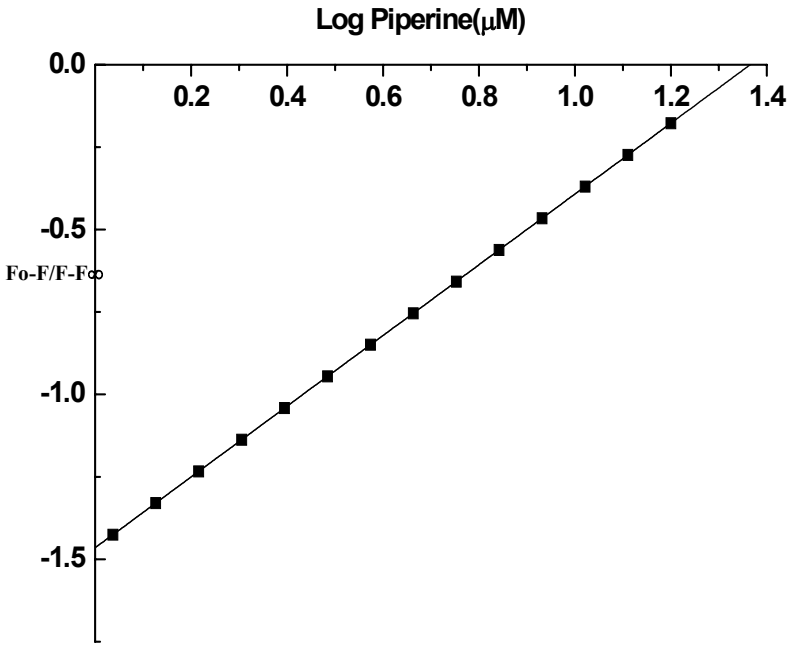


Fig. 3 *Stoichiometry plot of the interaction of HSA with piperine by fluorescence quenching.*

HSA (1μM) in 50 mM Tris-HCl buffer, pH 7.4 was titrated with increasing aliquots of stock piperine solution (2 μl equivalent to 1 μM piperine per aliquot) in methanol and the % quench was recorded. Fluorescence quench was corrected due to absorption of light by piperine as mentioned in the text. The results are average of five sets of independent experiments.

Table 1. Effect of temperature on Stern-Volmer Constant for HSA-Piperine interaction.

Temperature (K)	Stern-Volmer Constant Ksq M-1
290	8.7 X 104
300	6.17 X 104
310	5.07 X 104
320	4.5 X 104

HSA (15μM) in 50 mM Tris-HCl buffer (pH 7.4) was titrated with increasing aliquots of stock piperine solution (15 μM piperine per aliquot) in methanol and the fluorescence intensity was monitored

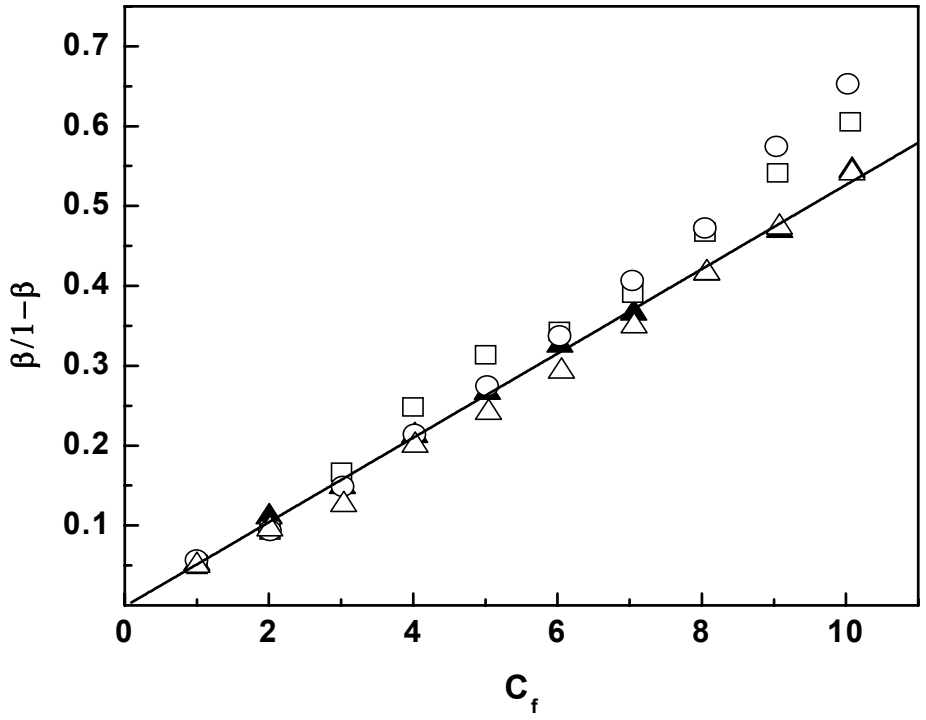


Fig. 4 *Effect of temperature on the binding constant of piperine to HSA : van't Hoff's plot.*

HSA (1μM) in 50 mM Tris-HCl buffer, pH 7.4 was titrated with increasing aliquots of stock piperine solution (2 μl equivalent to 1 μM piperine per aliquot) in 100% methanol at different temperatures (17°C, 27°C, 37°C and 47°C and the % quench was recorded. The quench was corrected for inner filter effect.

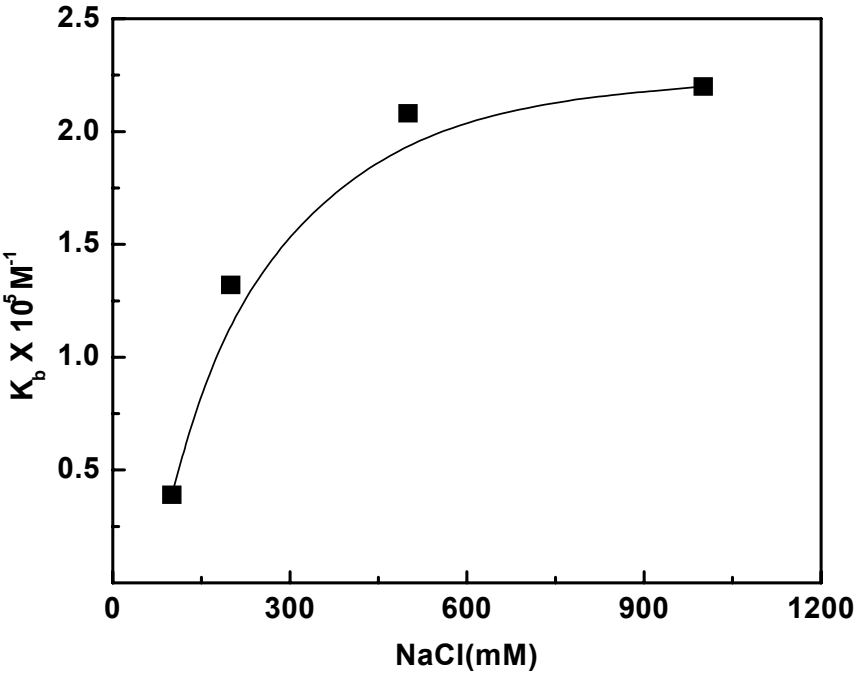


Fig 5 *Effect of Ionic Strength on the binding constant of piperine to HSA.*

A plot of the binding constant as a function of ionic strength to show the effect of ionic strength on the binding constant of piperine. HSA (1μM) in 50 mM Tris-HCl buffer, pH 7.4 was titrated at different ionic strengths adjusted by using sodium chloride (100 mM 200 mM, 500mM and 1000mM) with increasing aliquots of stock piperine solution (2 μl equivalent to 1 μM piperine per aliquot) in 100% methanol. The % quench of the intrinsic fluorescence of HSA was recorded. The quench was corrected for inner filter effect.

Fluorescence of albumin bound piperine and competition with other ligands

Piperine is intrinsically a weak fluorescent molecule. This property has been utilized to study the nature of binding to HSA. There was a shift of the emission maxima of albumin bound piperine towards shorter wavelength compared to unbound piperine which has emission maxima of 480 nm (Fig.6). This indicates that piperine is binding to the hydrophobic crevice of HSA.

The fluorescence of albumin bound piperine was not affected by the addition of warfarin (known binder to site II-A), diazepam (known binder to site III-A) or ANS (known II-A and III-A binder) (not shown in figure). On the other hand, the fluorescence of albumin bound piperine was affected by the addition of TIB, which is known to bind to sites II-A and I-B (Fig. 7). This implies that piperine and TIB compete for the same binding site on HSA. Addition of hemin (known I-B binder) results in the red shifting of piperine emission maxima from 440 nm to 480 nm, which is the emission maximum of unbound piperine (Fig. 8). Conversely, the addition of piperine to the complex of HSA with warfarin did not displace warfarin as indicated by the unperturbed anisotropy of warfarin (Fig. 10).

Binding distance

HSA when excited at 280 nm has an emission maximum at 333 nm. Piperine has absorption maxima at 345nm. The absorption spectra of piperine overlap with the emission spectra of serum albumin (Fig. 11). Addition of piperine quenches the fluorescence of serum albumin indicating efficient Förster type resonance energy transfer (FRET). There is a good overlapping between the fluorescence emission spectrum of free HSA and UV absorption spectra of piperine (Fig. 11). As the fluorescence emission of protein was affected by the excitation light around 280 nm,

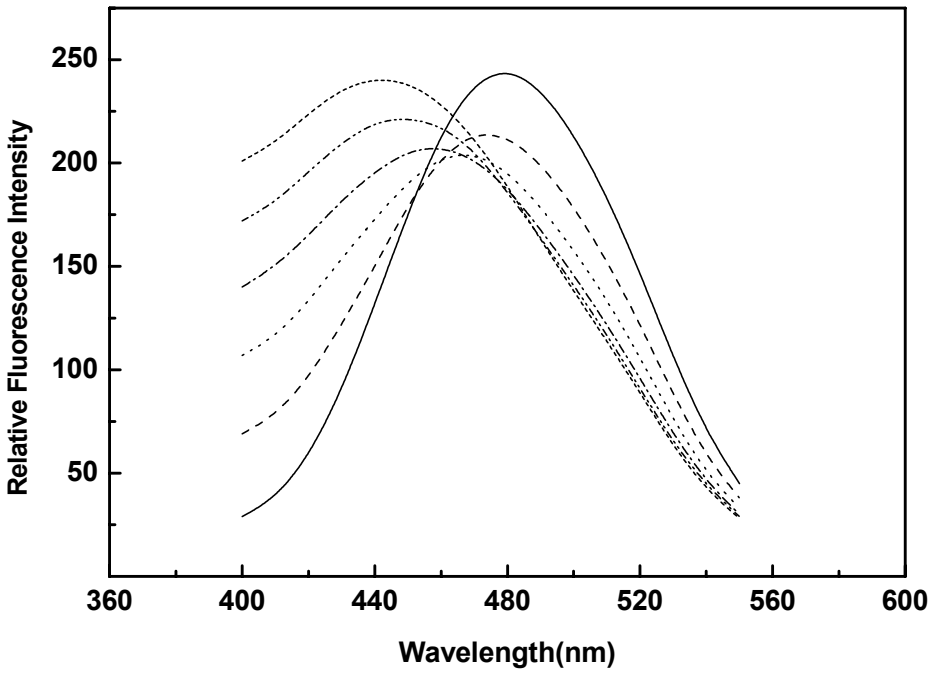


Fig. 6 *Emission Spectra of piperine showing blue shift on binding to HSA.*

Piperine (2.75 μM) in 50 mM Tris-HCl pH 7.4 was titrated against increasing concentrations of HSA in the same buffer. The final concentration of HSA was 14.75 μM. Stock HSA (835 μM) was added in 5 μl aliquots and the spectra recorded between 400 – 550 nm after excitation at 345 nm, the excitation maxima for piperine. Excitation slit width was 5 nm and emission slit width was 10nm.

..... Free piperine;
——— Piperine bound to HSA,
concentration of HSA is 14.75 μM.

the spectrum ranging from 300 to 450 nm was chosen to calculate the overlap integral. The calculated result shows that J is 2.69×10^{-15} cm^3 L mol^{-1}. The energy transfer efficiency E is 0.05. If the value of E, K^2, Φ and n are known, R_0 and r can be calculated. R_0 is found to be 1.7 nm and r is 2.79 nm. While the binding distance of piperine was more than their respective critical distance R_0, the fluorescence quenching was more likely induced by the static quenching other than non-radiative energy transferring.

Time resolved fluorescence studies

Bovine serum albumin, a two-tryptophan system (Trp – 134 and Trp – 212) was employed to establish the binding cleft of piperine on serum albumin. The time resolved fluorescence study revealed two sets of lifetime distributions for native BSA, one centered at 6.47 nanosecond (ns) contributing to 88% of the total fluorescence (Trp – 134) and the other centered at 2.89 ns (Trp – 212) contributing to the rest of the fluorescence. Addition of piperine to BSA causes a decrease in the amplitude of longer lifetime component (Fig.12B). The detailed analysis of the data has been carried out using equation-II. The data has been fitted to a bi-exponential function giving rise to lifetimes of 6.47 and 2.89 ns with amplitudes of 0.60 and 0.18 for the two tryptophans respectively. The χ^2 value for this fit was 1.029. To understand the localization and influence of piperine on lifetime, experiments have been carried out by varying the concentration of piperine and analyzing the fluorescence at each concentration. These results are summarized in Fig. 12A. It is observed that with the increase in the concentration of piperine, the lifetime of the longer lifetime component

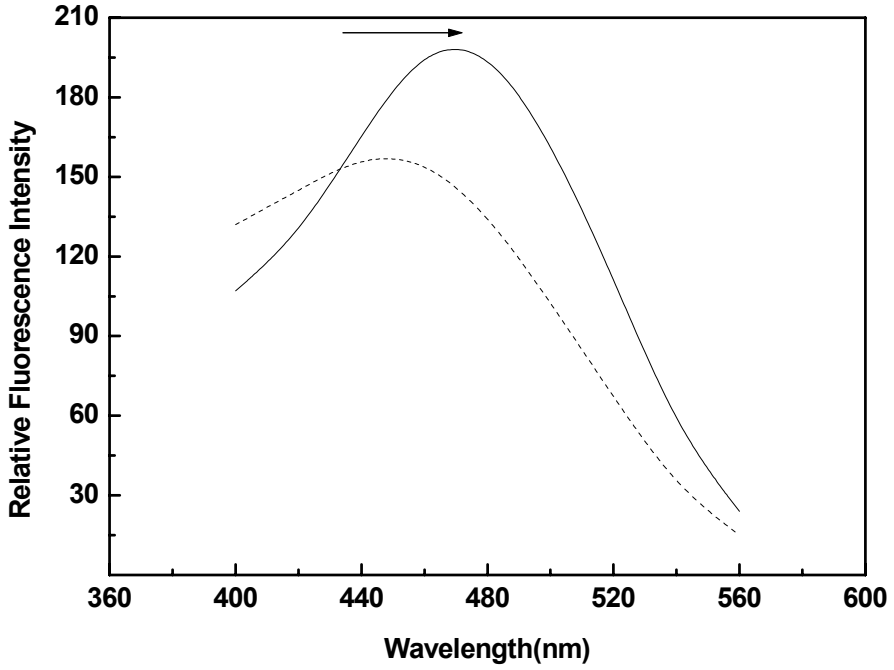


Fig. 7 *Competitive ligand interactions of HSA: piperine.*

Piperine (10μM) was complexed with HSA (10μM). The excitation wavelength was 345 nm and the emission wavelength range was from 400-550 nm respectively. Slit widths were at 10 and 10 nm for excitation and emission respectively. The arrow indicates the direction of fluorescence shift. 2μl of 10mM TIB in 100% DMSO was added. Temperature was maintained at 27°C using a water bath. …… Bound piperine; _____ Free piperine

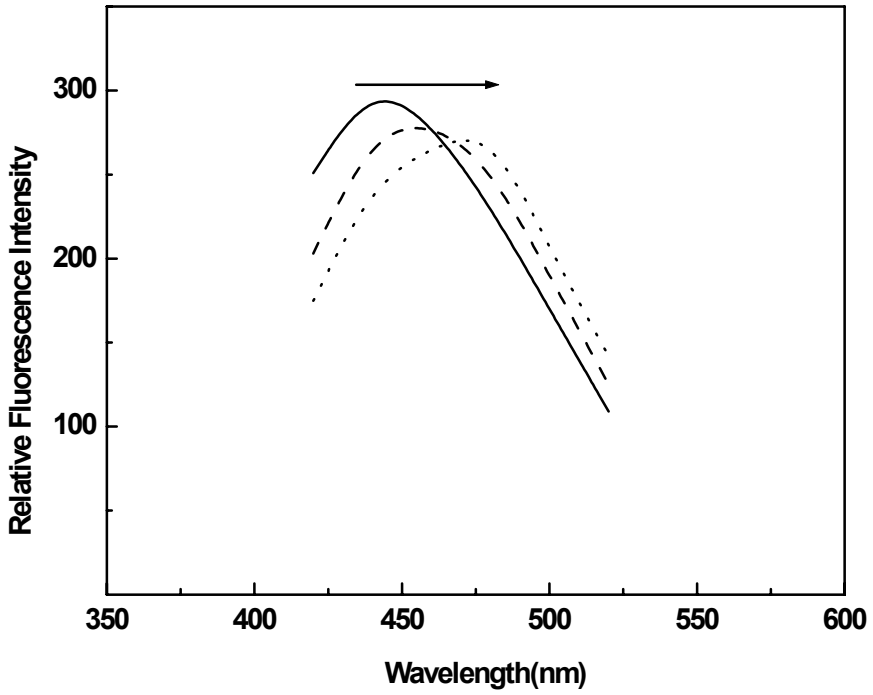


Fig 8 *Competitive ligand interactions of HSA: piperine.*

Piperine (10μM) was complexed with HSA (10μM). The excitation wavelength was 345 nm and the emission wavelength range was from _____ 400-550 nm respectively. Slit widths were at 10 and 10 nm for excitation and emission respectively. The arrow indicates the direction of fluorescence shift. 2μl of 10 mM hemin in 10mM NaOH was added for each addition.

_____ Piperine bound to HSA;

---------- Piperine bound to HSA in presence of 12.5 μM hemin;

..…… Free piperine in presence of 25 μM hemin

decreased significantly, whereas, lifetime of the shorter lifetime component did not vary appreciably. The average lifetime was calculated by the method of Sengupta *et al* (2003). Fig.12B is the plot of amplitude of different lifetime components against piperine concentrations. The amplitude of the longer lifetime component decreases from 0.60 to 0.12, with increase in piperine concentration. However, the amplitude of shorter lifetime component increases. Addition of Piperine to HSA did not alter the lifetime of tryptophan (Trp – 214) appreciably (Table 2).

Discussion

The most unique feature of serum albumin is its ability to reversibly bind to a wide variety of endogenous and exogenous bioactive compounds. Extensive studies have proved that albumin is the principal transport protein for fatty acids and other lipids that would otherwise be insoluble in the circulating plasma. Serum albumin comprises three homologous domains (I, II & III) as evident from the X-ray crystal structure (He & Carter, 1992). Each binding domain is a product of two sub-domains A and B with common structural motifs, which are predominantly helical and extensively cross-linked by several disulfide bridges. The principal regions of ligand binding to HSA are located in hydrophobic cavities in sub-domains II-A (binding site I) and III-A (binding site II). In each of the two sub-domains, there is an asymmetric distribution of amino acid residues leading to a hydrophobic surface on one side and a positively charged surface on the other (Carter & Ho, 1994).

From the present study, it is clear that piperine binds to HSA with a binding constant of $5.0 \pm 0.2 \times 10^4$ M^{-1}. The stoichiometry of the HSA-piperine complex is 1 : 1,

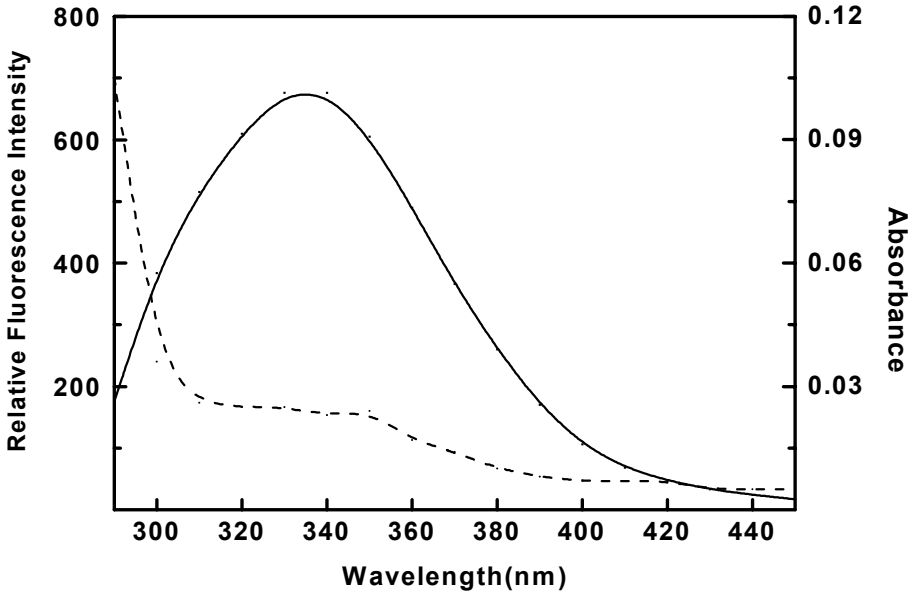


Fig. 9 *Resonance energy transfer from HSA to Piperine.*

Emission spectra of HSA in 50 mM Tris-HCl (pH 7.4). Excitation wavelength was 295 nm. Emission range was 300 - 400 nm with slit widths of 5 nm for excitation and 10 nm for emission. Protein concentrations used was 1 μM. Temperature was maintained at 27°C using a water bath.

———— Emission spectra of HSA.
---------- Absorption spectra of piperine.

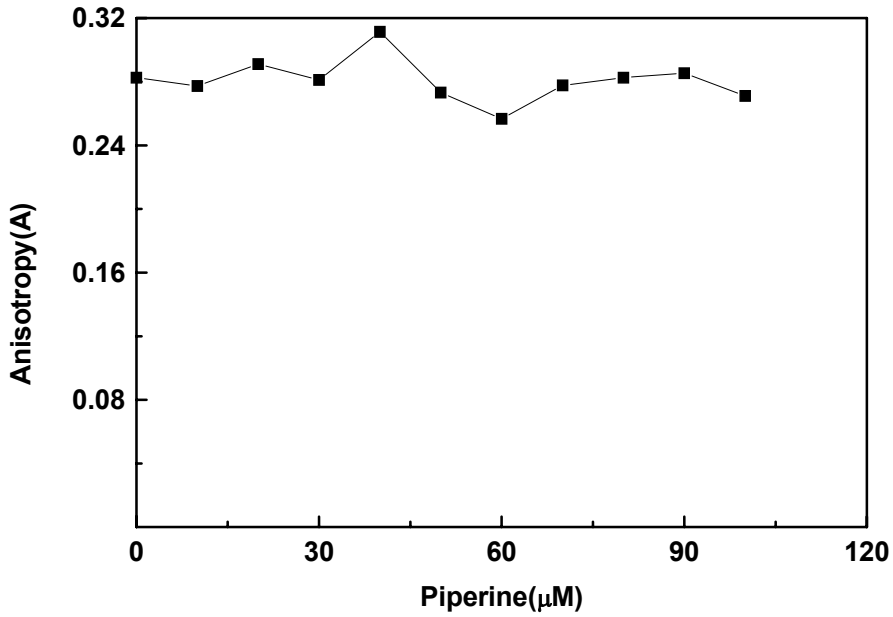


Fig. 10 *Variation in fluorescence anisotropy (A) of warfarin-HSA complex as a function of piperine concentration.*

Warfarin (10μM) was complexed with HSA (10μM). The excitation and emission wavelengths were 310 and 385 nm respectively. Slit widths were at 5 and 5 nm for excitation and emission respectively. To this complex 1μl of 25mM piperine in 100% methanol was added. Temperature was maintained at 27°C using a water bath.

suggesting that one mole of HSA binds one mole of piperine. The binding constant is invariant in the temperature range 17 - 47^0C suggesting that this interaction is hydrophobic in nature. The Stern-Volmer constant decreases with temperature indicating that quenching of HSA fluorescence by piperine is static in nature. Thus, it can be inferred that the formation of complex takes place in the ground state of the fluorophore. The binding constant increases with increase in ionic strength. The fluorescence of albumin bound piperine is incremental accompanied by a blue shift with a rise in concentration of HSA concentration, which confirms the binding of piperine in a hydrophobic cleft.

The distance between piperine and tryptophan in HSA-piperine was found to be 2.79 nm. There is a lone tryptophan residue in HSA, Trp214 and the distance from the bound ligands to Trp214 is less than 7 nm, which suggests that a non-radiative energy transfer mechanism may be among the quenching mechanisms.

The quenching of intrinsic fluorescence measurements of HSA and BSA assist in the identification of binding sites. In practice, tryptophan fluorescence is more commonly studied as tyrosine fluorescence is frequently very weak due to quenching. The fluorescence of tyrosine residue is almost totally quenched if it is located near the tryptophan. The binding crevice for piperine on HSA was identified by steady state and time resolved fluorescence measurements. ANS has two binding sites on HSA namely IIA and IIIA, with a binding constant of 7.9 $\times$ 10^4 M^{-1} and 8.7 $\times$ 10^5 M^{-1} respectively (Bagatolli *et al*, 1996). Addition of piperine did not quench the fluorescence of ANS-HSA complex, there by suggesting that piperine and ANS do not

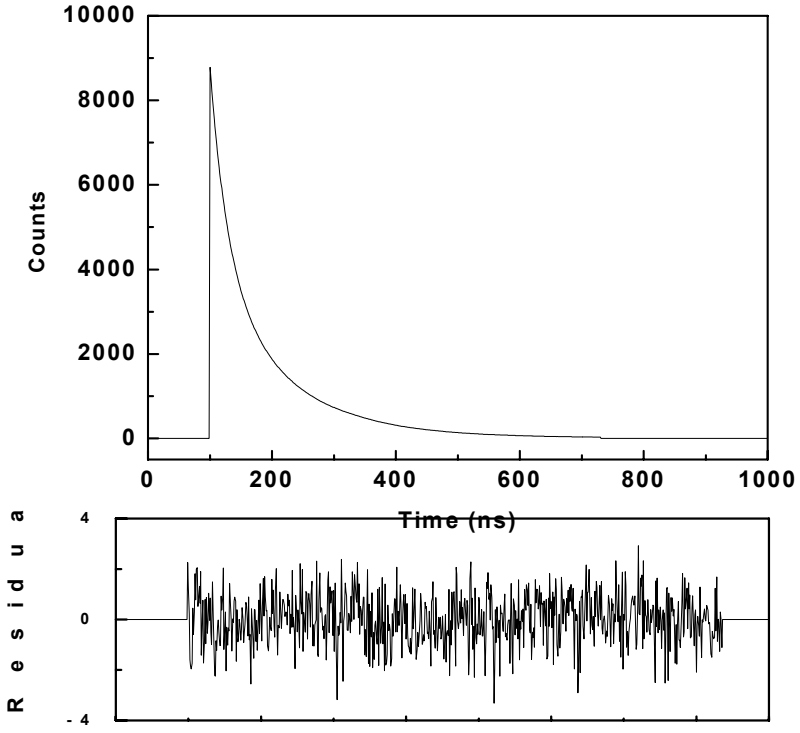


Fig. 11 *Bi-exponential fit of time resolved fluorescence decay of BSA*.

Lower curve shows the weighted residual illustrating the accuracy of the exponential fit

Table 2. Fluorescence decay characteristics of HSA

HSA : Piperine	Average Life time (ns)	χ^2
1 : 0	2.92	1.21
1 : 1	3.24	1.05
1 : 2	3.11	1.11
1 : 3	2.86	1.08

HSA (15μM) in 50 mM Tris-HCl buffer (pH 7.4) was titrated with increasing aliquots of stock piperine solution (15 μM piperine per aliquot) in methanol and the fluorescence decay was monitored.

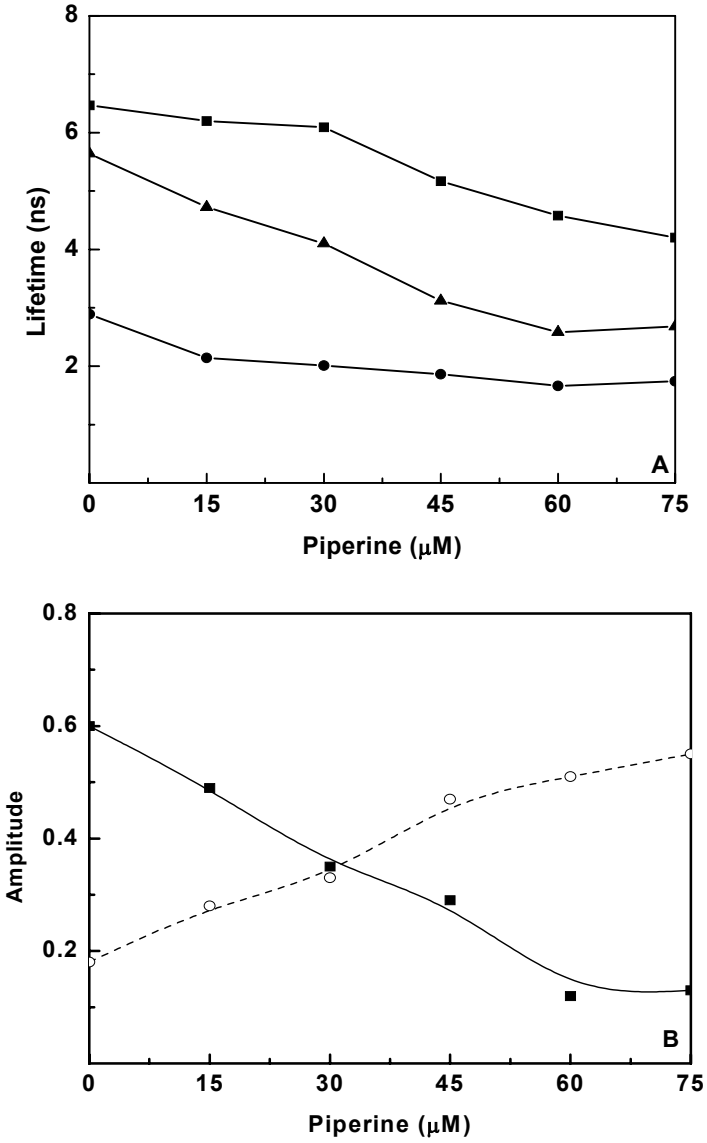


Fig. 12A. *The dependence of lifetime of BSA fluorescence on the concentration of piperine*.

Piperine was added to fixed concentration of BSA (15μM). - ▲ - Average life time; - ■ - Life time of the longer life time component; -● - Life time of the shorter life time component.

Fig. 12B *The dependence of amplitude of BSA fluorescence on the concentration of piperine*.

Piperine was added to fixed concentration of BSA (15μM). -■- Amplitude of longer life time component; - o - Amplitude of shorter life time component

compete for the same binding site on serum albumin. Warfarin is a marker ligand that binds to subdomain II-A of serum albumin (Petitpas *et al*, 2001). Neither addition of piperine to warfarin-HSA complex nor the addition of warfarin to piperine-HSA complex affects the fluorescence of the complex. In addition, warfarin anisotropy was also not affected by the piperine addition. This suggests piperine and warfarin do not compete for the same binding site on albumin. Diazepam, a characteristic marker ligand for sub domain III-A of serum albumin (Maruyana *et al*, 1993) also did not perturb the fluorescence of piperine in the piperine-HSA complex ruling out binding of piperine to sub domain III-A.

TIB is shown to bind to two well separated binding sites, sub domains II-A and I-B (Curry *et al*, 1998). Addition of TIB to piperine-HSA disrupts the bound piperine fluorescence. This observation confirms TIB and piperine compete for the same site on albumin. It is reported that hemin binds to a hydrophobic, D-shaped cavity in sub-domain I-B of serum albumin (Zunszain *et al*, 2003). Addition of hemin to piperine-HSA complex resulted in the shift of emission maxima of piperine from its bound form to free form. From these observations, it is clear that piperine is not binding to the sub-domain II-A and III-A as indicated by the unperturbed fluorescence properties when titrated against ANS (ligand for II-A and III-A), warfarin (ligand for IIA) and diazepam (ligand for III-A). On the other hand, displacement of piperine from its HSA complex by TIB and hemin indicates that piperine is binding to the site I-B of HSA.

To get more insight in to the binding of piperine to serum albumin, time resolved fluorescence technique was employed. BSA has 76 % homology with HSA (Peters, 1985). The difference between HSA and BSA with reference to fluorescence is an additional tryptophan at position 134 in site IB (Carter & Ho, 1994). The binding

constant of piperine is same with BSA and HSA indicating that both have equal affinity for piperine. Time resolved fluorescence study revealed that BSA has two sets of lifetime distributions. These two lifetimes have been assigned to the two tryptophans present in BSA (Patel *et al*, 1999) as the intense peak is the life time distribution pattern centered at 6.2 ns corresponds to Trp-134 and the other peak centered at 2.2 ns is due to Trp-212. Piperine addition to serum albumin resulted in a decrease of the average lifetime from 5.64 to 2.68 ns. This could be due to a decrease in the contribution of species with longer lifetime to total fluorescence intensity. Addition of piperine to BSA selectively decreased the amplitude of longer lifetime component confirming that piperine binds near the Trp-134 and quenches its fluorescence. The observed increase in the amplitude of Trp-212 is due to the constraint imposed on the sum of amplitudes, which should be unity when analyzed by Equation - V. The lifetime of tryptophan of HSA did not change appreciably with increase in piperine concentration implicating that Trp-214 is not directly involved in the recognition of this ligand.

The sub-domain I-B of HSA is a deep hydrophobic slot, which contains amino aids such as Tyr-138, Tyr-161, Leu-139, His-146, Ile-142 and Leu-154. While not all these hydrophobic residues of the potential hydrophobic residue line up might interact with piperine, some of these residues are likely to interact with piperine thus explaining the hydrophobic interaction. Thus, the observations of the competitive ligand binding studies strongly indicate that the binding site of piperine on serum albumin to be site I-B, which was further confirmed by time resolved fluorescence studies.

Piperine, the main bioactive alkaloid compound of the spice - black pepper is essentially hydrophobic, and hence necessitates a facilitated mechanism for its

transport in the blood circulation. Curcumin the bioactive principle of turmeric, which shares a considerable portion of structure with piperine, has been shown to bind predominantly to the site II-A of HSA (Zsila *et al*, 2003). Curcumin is also predicted to bind to a secondary site on serum albumin with lesser affinity. Thus, piperine and curcumin, in spite of having considerable structural homology bind to different sites on serum albumin. This difference in their binding sites may probably be attributed to the structural differences in the vanillyl moiety of the two ligands. Whereas, curcumin has methoxy and hydroxyl functional groups, piperine has methylene dioxy group replacing the former two attached to the benzene ring. Whereas, binding constant of curcumin for serum albumin is reported to be of the order of 10^5 M^{-1} (Reddy *et al*, 1999), piperine is observed in the current study to have a moderate binding constant of 10^4 M^{-1}. Moderate binding constant of piperine for serum albumin also explains the observed distribution of this compound in body tissues and its faster elimination within few days of its oral intake.

In conclusion, the results of this study clearly demonstrates that piperine binds to serum albumin reversibly by hydrophobic interactions with a binding constant of 10^4 M^{-1} having the stoichiometry of 1 : 1 preferably at the binding site I-B. Even though binding constant of piperine with serum albumin is moderate, major portion of the piperine in blood circulation may still be in the bound form due to very high physiological concentration of albumin (0.6 mM). Thus, serum albumin can act as a carrier for piperine in blood under physiological conditions to deliver this bioactive compound to the target tissues, where it elicits its pharmacological effect.

Section-B: Studies on the interaction of capsaicin with human serum albumin

Summary

In the present investigation, interaction of the spice principle – capsaicin with HSA has been examined employing spectroscopic measurements – fluorescence, size exclusion chromatography and NMR. Capsaicin, the bioactive alkaloid compound of the spice - red pepper exhibits a wide range of beneficial physiological and pharmacological activities. Being essentially water-insoluble, capsaicin is presumed to be assisted by serum albumin for its transport in blood. In this study, the binding of capsaicin to serum albumin was examined by employing fluorescence measurement, Size exclusion chromatography and NMR which suggested the binding of capsaicin with HSA. No further information on the binding of capsaicin to HSA could be drawn due to limitations such as overlapping fluorescence property of capsaicin with that of HSA, limited solubility of capsaicin in water, and lower molar absorption coefficient of capsaicin.

Materials and Methods

Materials

Human serum albumin (HSA), Bovine serum albumin (BSA), warfarin, diazepam, triiodobenzoic acid, hemin and Trizma base were from M/s Sigma Aldrich Chemical Co. (St. Louis, USA). Piperine and 8-anilino-1-naphthalene sulfonic acid (ANS) were from M/s Aldrich Chemical Co., Milwaukee, USA. All other chemicals used here were of analytical grade.

Purification of human and bovine serum albumin

The higher molecular weight aggregates associated with commercial preparations of human and bovine serum albumin were removed by size exclusion chromatography on a Sephadex G-100 column (120 X 1 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Fractions of 1 ml were collected at a flow rate of 10 ml/ h and the purity was ascertained by SDS-PAGE (Laemmli, 1970). Protein concentration of the HSA fractions was determined using a value of 5.30 for $E_{1\%}$ at 278 nm (Clark *et al*, 1962). BSA concentration was estimated using a value of 6.67 for $E_{1\%}$ at 279 nm (Föster & Sterman, 1956).

Flurorescence measurements

Serum albumin samples were centrifuged at 26,000 x g for 30 min to remove any aggregates. Stock solution (1.25 mM) of capsaicin in methanol was added in increments of 2 μl to 1 μM HSA in 0.05 M Tris-HCl, pH 7.4. Fluorescence quenching of HSA by capsaicin was followed at 27 ± 0.2°C. The excitation and emission wavelengths were set at 280 nm and 333 nm, respectively. Slit widths for excitation and emission were 5 and 10 nm, respectively. Blank titrations, with methanol, were carried out to correct the quenching due to methanol. The fluorescence readings were corrected for inner filter effects due to absorption of light by the addition of ligand to the protein-ligand complex (Ward, 1985).

Size exclusion chromatography

Capsaicin binding to HSA was monitored by Hummel – Dreyer method (1962).

A 0.7 X 12 cm column was packed with Biogel P-2. The column was equilibrated at least five bed volumes with buffer containing 50 μM capsaicin. 0.1 ml of 50 μM HSA was loaded on to the column and eluted with the same buffer which was used for the equilibration at 25^0C. Fractions (0.5 ml) of the eluted aliquots were collected in test tubes. Absorbance was measured at 280 nm (Absorption maxima of capsaicin). A plot of concentration of the eluent against fraction number was plotted.

NMR Studies

NMR experiments were performed at 25^0C on a Bruker Avance 500 MHz spectrometer using a 5 mm BBO probe equipped with z-gradient coil. Proton Saturation Transfer Difference (STD) NMR spectra were recorded using the STD sequence with presaturation of water signal. The offset of the saturation pulse on protein was changed between alternate scans to be at 100 Hz for on-resonance and 10,000 Hz for off-resonance with respect to external TMS. The STD spectra were collected with 180^0 phase change between the scans to remove the direct signal. Gaussian shaped pulse with width of 25 ms and power 50 Hz was used for saturating the protein peaks and a 100 μs inter pulse delay was kept between saturation. The offset of the hard pulse of 10.5 μs width was kept on the water peak.

Results and Discussion

Steady state fluorescence measurements

The binding of capsaicin with HSA has been monitored by following relative fluorescence intensity of serum albumin, and is presented in Fig.13. Addition of capsaicin to HSA did not quench the fluorescence of HSA. This is due to the overlapping of fluorescence properties of HSA with that of capsaicin. Hence, the

binding of capsaicin to serum albumin could not be monitored by fluorescence measurement method.

Size-exclusion chromatography

Since the fluorescence measurement method thus employed could not ascertain the binding of capsaicin with HSA, due to the limitation encountered, Size-exclusion chromatography technique was applied as an alternative for the quantitative characterization of the ligand (capsaicin) binding to HSA in terms of the stoichiometry and strength of the equilibrium phenomenon. Hummel – Dreyer method is one such chromatographic technique employed here in which the gel matrix is selected in such a way that the ligand is included and the protein is excluded. Additionally, the ligand (capsaicin) or the protein concerned did not interact with the matrix (Bio Gel P-2).

The column is equilibrated with known concentration of the ligand solution followed by the application of small volume of protein solution of known concentration and eluted with the same ligand solution. The elution profile was monitored at 280 nm (the absorption maxima of the ligand). Fig.14 presents the Hummel-Dreyer plot of capsaicin elusion profile. Appearance of a trough in the elution profile in this plot confirmed the binding of capsaicin with HSA. Although this method would provide for the determination of the binding constant by performing the experiment with varied concentrations of the ligand and protein, the binding of the ligand capsaicin to HSA was not quantifiable in terms of binding constant because of the following reasons. (a) This method is applicable to capsaicin only at very low concentrations, while at higher concentrations capsaicin was found to interact with the column material. (b) At lower concentrations, absorption at 280 nm of capsaicin is disadvantageously very less. Thus, this method could not be pursued for further studies.

NMR studies

The Nuclear Magnetic Resonance (NMR) technique was used to study the interaction between HSA and capsaicin. The NMR approaches used in protein - ligand interaction studies are of two classes depending on whether the resonances of the protein or the resonances of the ligand are monitored. The second case which relies on the observation of the ligand resonances is exclusively applicable to ligand in the moderate to low affinity range. The variables such as line broadening, change of Nuclear Overhauser Effect from positive to negative (transfer NOE), intermolecular magnetization transfer, restricted ligand diffusion, change in relaxation properties of the ligand due to interaction with spin labels etc., which are monitored during NMR experiments are indicative of ligand binding to protein.

Magnetization transfer can occur between protein and ligand spins transiently forming complexes through NOE. Such intermolecular magnetization transfer forms the basis of saturation transfer difference (STD) methods. In STD experiments, the target resonances are saturated selectively; ligand spins that come in contact with the target receive part of the receptor spin saturation which results in a decrease of their line intensities in a one dimensional spectrum. The fraction of ligand resonances that have experienced a decrease in intensity following saturation of the target spins identifies the binding epitope of the ligand.

Fig.15 and Fig.16 present the NMR spectra of free capsaicin and of HSA-bound capsaicin respectively. The broadening of the peaks of proton resonances of capsaicin indicates that capsaicin is binding to HSA (Fig.16). These proton resonance peaks could not however be used for quantitative integration, since they are broad. In addition to the above limitation (line broadening), further NMR studies involving ligand

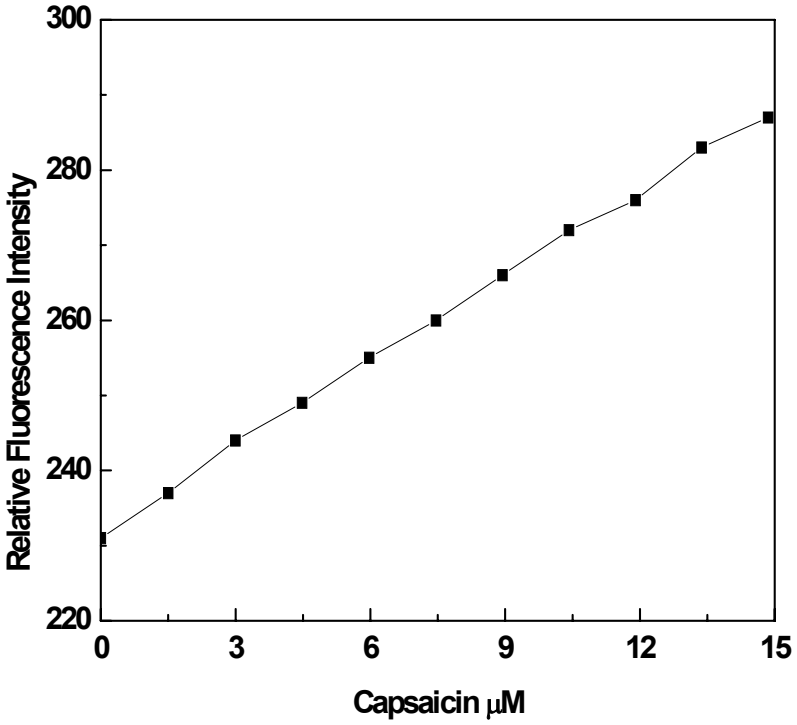


Fig. 13 *Interaction of capsaicin with HSA by fluorescence measurement*

HSA (1μM) in 50 mM Tris-HCl buffer (pH 7.4) was titrated with increasing aliquots of stock capsaicin solution (2 μl equivalent to 1.49 μM capsaicin per aliquot) in methanol and the fluorescence was recorded.

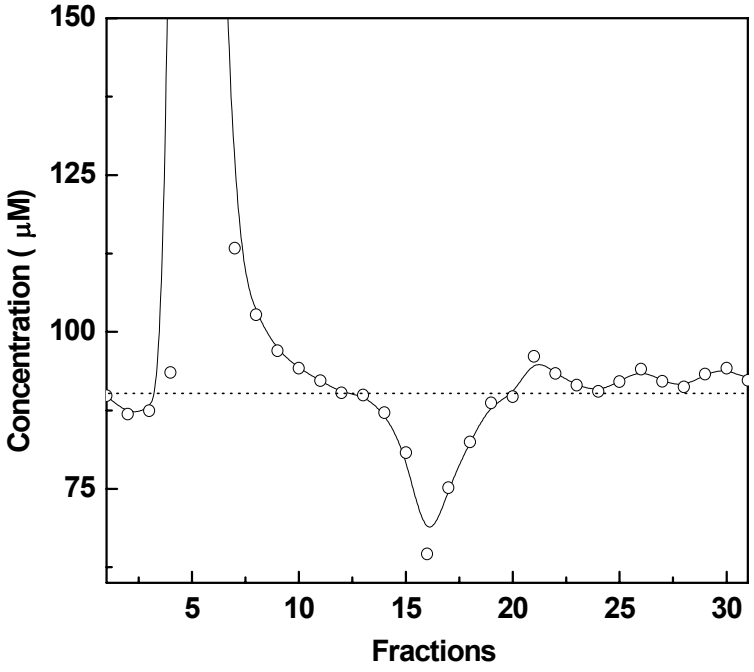


Fig.14 Hummel-Dreyer plot of capsaicin binding to HSA

Size exclusion (Elusion profile) was monitored at 280 nm using conventional chromatographic column containing Biogel P-2. The dotted line indicates the baseline absorbance of capsaicin in the buffer.

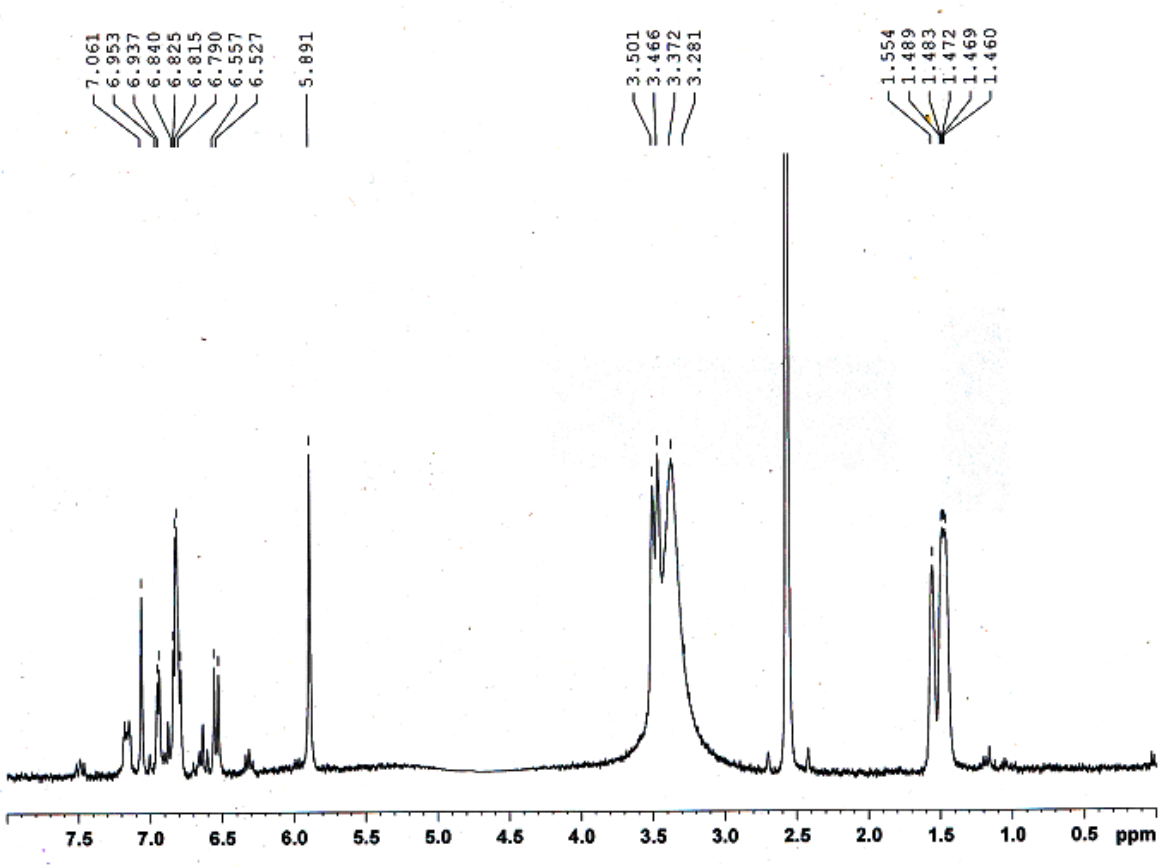


Fig. 15 STD NMR spectrum of free capsaicin

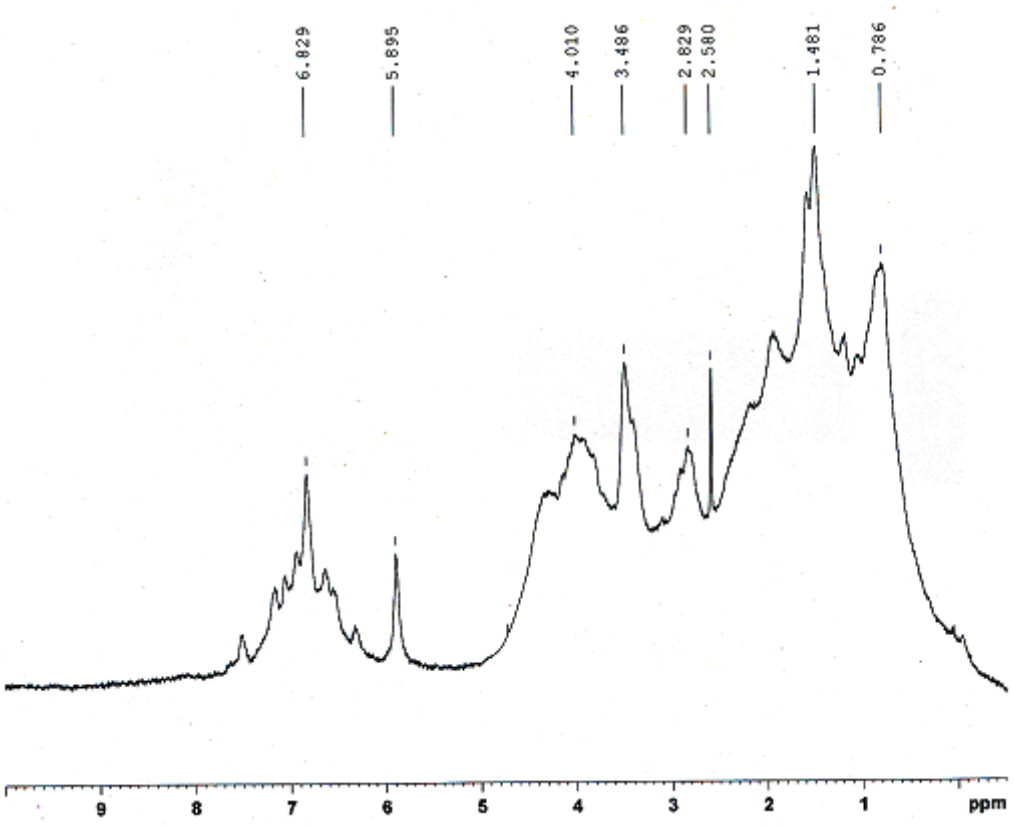


Fig.16 STD-NMR profile of HSA-bound capsaicin

monitoring could not be performed with higher ligand to protein ratio due to the limited solubility of capsaicin in water. Thus, it was not possible to predict which part of the ligand is interacting with the protein.

Equilibrium dialysis and Isothermal titration calorimetric methods could not be employed in this effort to study the interaction of capsaicin with HSA, as the aqueous solubility of capsaicin is very less and also it undergoes degradation with time. Thus, it was not possible to draw any more information on the interaction of the spice principle – capsaicin with serum albumin from our research efforts discussed above. Probably, this necessitates the use of Surface Plasmon Resonance technique and more sensitive HPLC detectors with advanced columns.

CHAPTER – V

INFLUENCE OF CURCUMIN, CAPSAICIN AND PIPERINE ON RAT LIVER DRUG METABOLIZING ENZYME SYSTEM *IN VIVO* AND *IN VITRO*

INFLUENCE OF CURCUMIN, CAPSAICIN AND PIPERINE ON RAT LIVER DRUG METABOLIZING ENZYME SYSTEM *IN VIVO* AND *IN VITRO*

Summary

The effect of dietary supplementation of spice active principles – curcumin (0.2%), capsaicin (0.015%) and piperine (0.02%) on the activities of liver drug metabolizing enzyme system was examined. All the three dietary spice principles significantly stimulated the activity of aryl hydroxylase. A synergistic action of dietary curcumin and capsaicin with respect to stimulating the activity of aryl hydroxylase was also evidenced when fed in combination. The activity of N-demethylase while essentially remained unaffected by dietary curcumin, capsaicin or their combination, was significantly lowered as a result of piperine feeding. UDP-glucuronyl transferase activity was decreased by dietary piperine and combination of curcumin and capsaicin. NADPH-Cytochrome C reductase activity was significantly decreased by dietary piperine. The levels of hepatic microsomal cytochrome P_{450} and cytochrome b_5 were not influenced by any of the dietary spice active principles. These spice active principles were also examined for their possible *in vitro* influence on the components of hepatic drug metabolizing enzyme system in rat liver microsomal preparation. Piperine significantly decreased the activity of liver microsomal aryl hydroxylase activity when included in the assay medium at 1×10^{-6} M, 1×10^{-5} M and 1×10^{-4} M level. Lowered activity of N-demethylase was observed in presence of capsaicin or piperine at 1×10^{-6}M in the assay medium. Hepatic microsomal glucuronyl transferase activity was significantly decreased *in vitro* by addition of capsaicin or piperine. Capsaicin and piperine brought about significant decrease in liver microsomal cytochrome P_{450} when included at 1 x

10^{-6} M and 1 x 10^{-5} M level, the effect being much higher in the case of piperine. The results suggested that while the three tested spice principles have considerable similarity in structure, piperine is exceptional in its influence on liver drug metabolizing enzyme system. The study also indicated that a combination of curcumin and capsaicin does not produce any significant additive effect on liver drug metabolizing enzyme system.

Introduction

Spices form an important class of food adjuncts consumed widely to enhance the sensory quality of foods, especially flavour, aroma and colour. In recent decades, a few common spices have been experimentally understood to impart several health beneficial physiological effects (Srinivasan, 2005). These include the digestive stimulant action, hypolipidemic effect, antidiabetic influence, antilithogenic property, antioxidant potential, anti-inflammatory property, anti-mutagenic and anti-carcinogenic potential. Among these, the hypolipidemic and antioxidant influences of dietary spices have far-reaching health implications.

The complex of enzymes present in the endoplasmic reticulum of mammalian cells known as Mixed Function Oxygenase System (MFOS) is involved in the hydroxylation of endogenous steroids besides its involvement in the primary metabolism of foreign compounds. A few spices are known to enhance catabolism of cholesterol to bile acids (Srinivasan *et al*, 2004), which essentially involves hydroxylation of cholesterol by hepatic MFOS. Information on the influence of active principles of spices on MFOS might offer a possible explanation to their observed physiological effects related to metabolism of cholesterol, bile acids and steroid hormones. Dietary antioxidant spices are now understood to protect laboratory animals against the induction of tumours by a

variety of chemical carcinogens (Srinivasan, 2005). Among spices, turmeric which contains curcumin is an active antimutagen both *in vitro* and *in vivo* (Joe *et al*, 2004). Protection against chemical carcinogenesis could be mediated via-antioxidant-dependent induction of detoxifying enzymes. Inhibition of chemical carcinogenesis may be brought about directly by scavenging the reactive substances or indirectly by promoting mechanisms which enhance detoxification.

In this study, we have investigated the effect of dietary supplementation of spice active principles - curcumin (of turmeric), capsaicin (of red pepper), combination of curcumin and capsaicin, and piperine (of black pepper) on the activities of drug metabolizing enzymes. In the present study, these spice active principles were also examined for their possible *in vitro* influence on the components of hepatic MFOS in rat liver microsomal preparation.

Materials and Methods

Curcumin (Diferuloyl methane) and synthetic capsaicin (N-vanillyl-6-nonanamide) were obtained from M/s. Fluka Chemie, Switzerland. Piperine (1-Piperoyl piperidine) was from M/s Aldrich Chemical Co., Milwaukee, USA. All other chemicals used here were of analytical grade.

Animal treatment

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort in accordance with the guidelines laid down by the NIH, USA, regarding the care and use of animals for experimental procedures and with due clearance from the Institutional Animal Ethics Committee. Adult male Wistar rats (8 per group) weighing 100-105 g were maintained on specific semi-synthetic diets for 10 weeks.

The basal diet comprised of (%): casein, 21; cane sugar, 10; corn starch, 54; refined peanut oil, 10; Bernhart-Tommarelli modified Salt mixture, 4 and NRC vitamin mixture, 1. Spice principles – curcumin (0.2 %), capsaicin (0.015%), piperine (0.02%), and curcumin (0.2 %) + capsaicin (0.015%) were included in this basal diet (as solution in refined peanut oil) to give various experimental diets. The animals were housed in individual stainless steel cages and had free access to food and water. The diet consumption and the gain in body weight during the experimental regimen in all spice groups were comparable to controls. For *in vitro* study, adult male Wistar rats weighing 200 - 210 g acclimatized to basal control semisynthetic diet (described above) for 1 week were used.

Liver microsome preparation

The animals were sacrificed by decapitation and livers were quickly excised, perfused with ice-cold physiological saline and chilled in ice. All subsequent operations were carried out at 0-4°C. One portion of the liver tissue was homogenized in 4 volumes of 0.25M sucrose in 0.01M phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer, while the other in 4 volumes of isotonic KCL (0.155M in 0.035M Tris-HCl buffer, pH 7.5). The homogenates were centrifuged at 14,000 x g for 15 min in Sorvall (RC-5B) super speed centrifuge, and the supernatant fraction at 100,000 x g for 60 min in an Ultracentrifuge (Beckman L2-65B) to sediment microsomes. The microsomal pellet was resuspended in0.25M sucrose or 0.05M potassium phosphate buffer (pH 7.5) respectively (microsome from 1g liver in 1 ml). Isotonic KCl was employed instead of 0.25M sucrose to minimize the adsorption of hemoglobin (if any) on microsomes. This preparation was used in the assay of cytochrome P_{450}, cytochrome

b_5, NADPH-cytochrome C reductase and NADH-cytochrome C reductase. In all other assays, microsomes from liver homogenized in sucrose medium were used.

Analytical methods

Aryl hydroxylase activity with aniline as substrate was assayed by the method of Shimazu (1965) by measuring the p-aminophenol formed during the reaction. N-Demethylase activity was assayed according to Pettit and Ziegler (1963) using N,N-dimethylaniline as substrate and estimating formaldehyde formed after aerobic incubation. UDP-Glucuronyl transferase activity with p-nitrophenol as the acceptor was measured by the method of Isselbacher *et al*. (1962). NADPH-Cytochrome C reductase and NADH-Cytochrome C reductase were assayed spectrophoto-metrically by measuring the rate of reduction of cytochrome C at 550 nm as described by Masters *et al*. (1967).

Cytochrome P450, the carbon monoxide binding pigment of liver microsomes was assayed by its carbon monoxide difference spectrum after reduction with sodium dithionine as described by Omura and Sato (1964). Microsomal protein was measured by the Lowry's procedure (1951) using bovine serum albumin as reference. Microsomal phospholipid was extracted by Folch's procedure (1957) and estimated according to Charles and Stewart (1980) using ammonium ferrothiocyanate.

Inclusion of spice principles in the in vitro study

Stock solutions of spice principles capsaicin and piperine were prepared in acetone. Suitable aliquots of these stock solutions were included in the assay systems of various MFOS parameters to give a final concentration of 1×10^{-6} M, 1×10^{-5} M and 1

x 10^{-4} M. All assays were conducted in duplicates from six independent liver microsomal samples.

Statistical analysis

Statistical evaluation of analytical data was done by Students' t-test and a 'p' value of <0.05 was considered significant (Snedecor & Cochran, 1976).

Results

Influence of dietary spice active principles on hepatic mixed function oxygenase system in rats are presented in Tables 1-3. All the three dietary spice principles – curcumin, capsaicin and piperine significantly stimulated the activity of aryl hydroxylase (Table-1). The enzyme activity was higher by 57, 51 and 90 % in these respective groups compared to control. Interestingly, aryl hydroxylase activity was even much higher (196 %) in the dietary curcumin + capsaicin combination group. This suggests the synergistic action of dietary curcumin and capsaicin with respect to stimulating the activity of aryl hydroxylase. The activity of N-demethylase while essentially remained unaffected by dietary curcumin, capsaicin or their combination, was significantly lowered as a result of piperine feeding (Table-1). The decrease in this enzyme activity brought about by dietary piperine was as much as 57 %.

Hepatic microsomal UDP-glucuronyl transferase activity was decreased by 36% by dietary piperine (Table-1). While this enzyme activity was not influenced by dietary curcumin and capsaicin independently, their combination had a negative influence (44% decreased activity). Hepatic microsomal NADPH-Cytochrome C reductase activity was significantly decreased by dietary piperine, the effect being 26% (Table-2).

Table 1. Influence of dietary spice active principles on hepatic mixed function oxygenase system in rats.

Animal group	Aryl hydroxylase[1]	N-Demethylase[2]	Glucuronyl transferase[3]
Control	28.4 ± 2.92	28.8 ± 2.86	63.8 ± 2.33
Curcumin	44.6 ± 3.50*	33.7 ± 1.78	57.4 ± 4.53
Capsaicin	42.8 ± 4.35*	36.5 ± 4.68	64.3 ± 4.63
Piperine	54.0 ± 5.50*	12.3 ± 0.53**	40.9 ± 4.32**
Curcumin + Capsaicin	84.5 ± 1.86*	31.0 ± 2.49	36.0 ± 4.30**

Values are mean ± SEM of 8 rats in each group.

Specific activity units: 1: nmole p-aminophenol formed / h / mg protein
2: nmole formaldehyde formed / h / mg protein
3: μmole p-nitrophenol conjugated / h / mg protein

*Significant increase compared to control.
**Significant decrease compared to control

Table 2. Influence of dietary spice active principles on hepatic mixed function oxygenase system in rats.

Animal group	NADPH-Cytochrome C Reductase	NADH-Cytochrome C reductase
Control	129.7 ± 9.55	433.1 ± 27.3
Curcumin	112.1 ± 7.28	444.4 ± 25.5
Capsaicin	114.3 ± 6.55	412.9 ± 13.0
Piperine	95.4 ± 3.56**	436.7 ± 21.7
Curcumin + Capsaicin	125.3 ± 7.05	415.0 ± 13.9

Values are mean ± SEM of 8 rats in each group.

Specific activity units: nmole cytochrome-C reduced / min / mg protein

**Significant decrease compared to control

Dietary curcumin and capsaicin either independently or in combination did not influence the activity of NADPH-Cytochrome C reductase. The activity of liver microsomal NADH-Cytochrome C reductase was not influenced by any of the dietary spice principles. The levels of hepatic microsomal cytochrome P_{450} and cytochrome b_5 were not influenced by any of the dietary spice active principles (Table-3).

The *in vitro* influence of capsaicin and piperine on the components of rat liver mixed function oxygenase system is presented in Tables 4 and 5. The spice principles were included in the assay medium at three concentrations, namely, 1×10^{-6} M, 1×10^{-5} M and 1×10^{-4} M. Liver microsomal aryl hydroxylase activity was significantly stimulated by capsaicin at the lowest concentration examined (1×10^{-6} M). The extent of increase in this enzyme activity was 38%. However, aryl hydroxylase was decreased by capsaicin (by 22%) when included in the assay medium at 1×10^{-5} M concentration. Piperine significantly and uniformly decreased the activity of liver microsomal aryl hydroxylase activity by 25 - 29% when included in the assay medium at 1×10^{-6} M, 1×10^{-5} M and 1×10^{-4} M level.

Slight but significantly lower activity of liver microsomal N-demethylase activity was observed in presence of capsaicin or piperine at 1×10^{-6}M in the assay medium, which was around 18 and 17% respectively (Table-4). Higher concentration of these spice principles in the assay medium did not have any influence on the enzyme activity. Hepatic microsomal glucuronyl transferase activity was significantly decreased *in vitro* by addition of capsaicin to the assay medium. The decrease in the enzyme activity was as high as 48 and 52% at 1×10^{-6} M capsaicin and 1×10^{-5} M capsaicin

Table 3. Influence of dietary spice active principles on hepatic mixed function oxygenase system in rats.

Animal group	Cytochrome P_{450} (nmole / mg protein)	Cytochrome b_5 (nmole / mg protein)
Control	0.748 ± 0.024	0.308 ± 0.020
Curcumin	0.811 ± 0.028	0.286 ± 0.012
Capsaicin	0.727 ± 0.014	0.264 ± 0.015
Piperine	0.683 ± 0.030	0.268 ± 0.019
Curcumin + Capsaicin	0.770 ± 0.016	0.264 ± 0.014

Values are mean ± SEM of 8 rats in each group.

Table 4. *In vitro* effect of spice principles on activities of liver microsomal aryl hydroxylase, N-demethylase and glucuronyl transferase

Concentration in the assay medium		Aryl hydroxylase[1]	N-Demethylase[2]	Glucuronyl transferase[3]
Control		83.8 ± 2.68	26.1 ± 0.51	123.8 ± 9.45
Capsaicin	1 X 10^{-6} M	116.0 ± 8.36*	21.4 ± 0.39**	64.4 ± 5.84**
	1 X 10^{-5} M	65.5 ± 2.46**	26.2 ± 0.94	59.5 ± 5.16**
	1 X 10^{-4} M	77.4 ± 6.39	25.7 ± 0.77	94.9 ± 10.5
Piperine	1 X 10^{-6} M	62.5 ± 4.65**	21.7 ± 0.49**	90.7 ± 7.00**
	1 X 10^{-5} M	59.1 ± 2.75**	24.9 ± 0.70	98.1 ± 5.86**
	1 X 10^{-4} M	60.7 ± 4.02**	25.6 ± 0.72	107.8 ± 7.78

Values are mean ± SEM of 8 values in each group.

Specific activity units: 1: nmole p-aminophenol formed / h / mg protein
2: nmole formaldehyde formed / h / mg protein
3: μmole p-nitrophenol conjugated / h / mg protein

**Significant decrease compared to control
* Significant increase compared to control

respectively. However, capsaicin when added at 1 x 10^{-4} M level, the lowered enzyme activity was however not statistically significant. Piperine significantly decreased the activity of hepatic microsomal glucuronyl transferase activity by 27 and 21% when included at 1 x 10^{-6} M and 1 x 10^{-5} M level in the assay medium respectively. The lowered activity of the enzyme by piperine at still higher concentration, namely 1 x 10^{-4} M was however not statistically significant.

While hepatic microsomal NADPH-cytochrome C reductase activity was not affected *in vitro* by either capsaicin or piperine at any of the three concentrations examined, the activity of NADH-cytochrome C reductase was slightly but significantly lowered by piperine at 1 x 10^{-6} M and 1 x 10^{-5} M level. The decreases in enzyme activity were 14 and 9% respectively. Capsaicin brought about 18 and 16% decrease in liver microsomal cytochrome P_{450} when included at 1 x 10^{-6} M and 1 x 10^{-5} M level (Table-5). Piperine brought about even higher decreases in liver microsomal cytochrome P_{450} when included at similar concentrations in the assay medium. The decreases observed in liver microsomal cytochrome P_{450} by piperine were 67 % and 65 %.

Discussion

The dosages of dietary curcumin, capsaicin and piperine employed here in this animal study roughly correspond to 5-times the calculated dietary intake of respective spices in the form of curry powder by Indian population (Thimmayamma *et al*, 1983). Hydroxylation of endogenous steroids, chiefly cholesterol, bile acids and steroid hormones is believed to be carried out by the same mixed function oxygenase system

Table 5. In vitro effect of spice principles on activities of liver microsomal NADPH-Cytochrome reductase, NADH-Cytochrome reductase and Cytochrome P450

Concentration in the assay medium		NADPH-Cytochrome reductase[1]	NADH-Cytochrome reductase[1]	CytochromeP450 (nmole/mg protein)
Control		123.4 ± 5.11	309.0 ± 7.78	0.818 ± 0.035
Capsaicin	1 X 10^{-6} M	123.9 ± 4.36	306.6 ± 9.47	0.669 ±0 .055**
	1 X 10^{-5} M	119.9 ± 5.03	306.8 ± 8.48	0.688 ± 0.042**
	1 X 10^{-4} M	115.8 ± 5.31	306.1 ± 11.0	ND
Piperine	1 X 10^{-6} M	122.7 ± 5.61	263.7 ± 5.31**	0.267 ±0 .022**
	1 X 10^{-5} M	122.6 ± 5.61	282.0 ± 7.19**	0.290 ± 0.029**
	1 X 10^{-4} M	117.4 ± 4.59	291.6 ± 9.72	ND

Values are mean ± SEM of 8 values in each group.

Specific activity units: 1: nmole cytochrome-C reduced / min / mg protein

**Significant decrease compared to control
ND: Not determined

of hepatic endoplasmic reticulum which is involved in the primary metabolism of foreign compounds. Hence, substances which alter monooxygenase activity may also influence cholesterol and bile acid hydroxylation. Conversion of cholesterol to bile acids is a multi-step process, where the initial step, viz., 7α-hydroxylation is the rate-limiting reaction. It is possible that spice principles fed animals wherein liver microsomal aryl hydroxylase activity is stimulated, cholesterol-7α-hydroxylase is also similarly activated. With this activation of the rate limiting enzyme, increased conversion of cholesterol to bile acids is likely to result. Higher activity of hepatic cholesterol-7α-hydroxylase has been evidenced in animals fed curcumin or capsaicin, but not in animals fed black pepper (Srinivasan & Sambaiah, 1991). This is in perfect agreement with the well documented hypochoelsterolemic potential of both curcumin and capsaicin and not of piperine or its parent spice - black pepper (Srinivasan *et al*, 2004). Increased bile acid secretion has been reported in animals fed spice principles – curcumin and capsaicin but not in piperine fed animals (Platel & Srinivasan, 2004).

Significant increase in the activity of hepatic microsomal aryl hydroxylase by dietary curcumin, capsaicin and piperine in adult female rats fed these compounds for 4 weeks has been reported (Sambaiah & Srinivasan, 1989). The present observation of the influence on cytochrome P_{450}-dependent aryl hydroxylase enzyme activity in male rats maintained on dietary spice principles for 8 weeks not only concurs with the earlier report, but has further evidenced that dietary curcumin and capsaicin exert a synergistic action on this enzyme activity. The enhancing influence of the combination of the two spice principles was roughly four times the effect of either of the individual spice principle. This stimulation of microsomal aryl hydroxylase by dietary spice principles was however without any similar effect on cytochrome P_{450}. It is also

intriguing that dietary piperine produced maximum stimulation of microsomal aryl hydroxylase activity in spite of causing a significant lowering of the activity of NADPH-Cytochrome C reductase. The reduction of cytochrome P_{450} catalysed by the flavoprotein NADPH-Cytochrome reductase is the rate-limiting reaction in microsomal drug metabolism. It is possible that hydroxylation of aniline (the substrate employed here for measuring aromatic hydroxylation activity) requires the participation of a different species of cytochrome P_{450}, probably cytochrome P_{448}. The significant decrease in cytochrome P_{450}-dependent N-demethylase activity brought about by dietary piperine alone is consistent with its similar effect on the activity of NADPH-cytochrome C reductase. Decreased activity of liver microsomal N-demethylase activity by dietary piperine has been earlier reported in adult female rats fed for 4 weeks (Sambaiah & Srinivasan, 1989).

Capsaicin, the pungent principle of red pepper has been shown to interact with hepatic drug metabolizing system *in vitro* with high affinity suggesting that it may be a potent inhibitor of biotransformation system (Miller *et al*, 1983). However, capsaicin *in vivo* has not inhibited any of the MFOS parameters examined here, while it has even enhanced the aryl hydroxylase activity. Piperine has been reported to inhibit hepatic monoxygenases and UDP-glucuronyl transferase *in vitro* in a dose-dependent manner (Atal *et al*, 1985). Contrary to the *in vitro* inhibition of aryl hydrocarbon hydroxylase, ethylmorphine N-demethylation, 7-ethoxycoumarin o-deethylation and 3-hydroxy-benzopyrene glucuronidation reported by these authors, Shin and Woo (1985) have observed that oral administration of piperine (100 mg/kg for 1 week) to mice increases hexobarbital hydroxylase, aminopyrene N-demethylase and the level of cytochrome P_{450}. Both capsaicin and piperine have been shown to produce type-I spectral change

in rat hepatic microsomes and to be competitive inhibitors of ethylmorphine or aminopyrene N-demethylation (Miller *et al*, 1983; Atal *et al*, 1985). In the current study, piperine *in vivo*, although has inhibited both N-demethylase and NADPH-cytochrome C reductase activities, it has stimulated aryl hydroxylation of aniline.

Curcumin feeding to mice has been reported to result in a considerable enhancement in the activity of phase-II drug metabolizing enzyme - glutathione-S-transferase to 1.7-times in liver as compared with corresponding normal diet fed control (Iqbal *et al*, 2003). The induction of such detoxifying enzymes by curcumin suggests its potential role as protective agent against chemical carcinogenesis and other forms of electrophilic toxicity. The significance of stimulation of phase-II detoxifying enzymes has been implicated in cancer chemopreventive effects of curcumin against the induction of tumours in various target organs. In the current study, however glucuronyl transferase - another phase-II detoxifying enzyme was found to be not favourably stimulated by dietary curcumin. Goud *et al*, (1993) who evaluated the effects of turmeric on xenobiotic metabolising enzymes in hepatic tissue of rats maintained on 0.5 to 10% turmeric for 4 weeks, observed significant elevation in the activities of UDP-glucuronyl transferase and glutathione-S-transferase at the highest dietary level. Interestingly in the current study, dietary combination of curcumin and capsaicin produced a significant decrease in the activity of hepatic microsomal glucuronyl transferase activity, which was not affected by the two individual spice principles.

Piperine, the alkaloidal constituent of black pepper is now established as a bioavailability enhancer of various structurally and therapeutically diverse drugs and other substances. Potential of piperine to increase the bioavailability of drugs in humans is of great clinical significance. The mechanism responsible for its

bioavailability enhancing action is understood to be either through protecting the drug from being metabolized in its first passage through the liver after being absorbed, or by promoting rapid absorption from the gastrointestinal tract, or by a combination of these two mechanisms. Piperine has been understood to be a potent inhibitor of drug metabolism by several independent investigations. In the context of piperine's ability to enhance drug bioavailability, Atal *et al,* (1985) studied the interaction of piperine with drug biotransforming reactions in hepatic tissue. Pretreatment with piperine prolonged hexobarbital sleeping time and zoxazolamine paralysis time in mice, while single oral administration of piperine in rats strongly inhibited hepatic UDP-glucuronyl transferase activity. Our present study also has evidenced significant decrease in hepatic microsomal glucuronyl transferase activity by dietary piperine.

Dalvi and Dalvi (1991) have observed that intragastrically administered piperine (100 mg/kg) increases hepatic microsomal cytochrome $P_{450,}$ cytochrome b_5, NADPH-cytochrome-C reductase, benzphetamine N-demethylase, aminopyrine N-demethylase and aniline hydroxylase at 24 h following treatment. However, intragastrically administered piperine at 800 mg/kg, produced a significant decrease in the levels of cytochrome P_{450}, benzphetamine N-demethylase, aminopyrine N-demethylase and aniline hydroxylase 24 h after treatment.

The modifying potential of black pepper on the hepatic biotransformation system has been assessed in mice fed on a diet containing 0.5, 1 and 2 % black pepper for 10 and 20 days (Singh & Rao, 1993). Data revealed a significant and dose-dependent increase in glutathione-S-transferase, cytochrome b_5 and cytochrome P_{450} in the

experimental groups. Being a potential inducer of detoxication system, the possible chemopreventive role of black pepper in chemical carcinogenesis is suggested.

Study of the *in vitro* influence of spice principles on various components of hepatic microsomal drug metabolizing enzyme system was limited to only capsaicin and piperine in this investigation. Curcumin was not studied here as it would interfere with the methods of assay due to its strong yellow colour. In this study, piperine has been evidenced to produce significant reductions in the activities of aryl hydroxylase, N-demethylase and glucuronyl transferase *in vitro* especially at concentrations of 1×10^{-6} M and 1×10^{-5} M in the assay medium, while at still higher concentration viz., 1×10^{-4} M the inhibitory effect was less evident. Capsaicin too was found to have inhibitory effect on all these three enzymes especially at concentrations of 1×10^{-6} M and 1×10^{-5} M in the assay medium.

Piperine inhibited aryl hydrocarbon hydroxylation, ethylmorphine-N-demethylation, 7-ethoxycoumarin-O-deethylation and 3-hydroxybenzo(α)pyrene glucuronidation in rat liver post-mitochondrial supernatant *in vitro* in a dose-dependent manner (Atal *et al*, 1985). Piperine's inhibition of these reactions in liver post-mitochondrial supernatant from 3-methylcholanthrene- and phenobarbital-treated rats was similar to the controls. Inhibition by piperine of aryl hydrocarbon hydroxylase (AHH) from 3-methylcholanthrene-treated rats was comparable to that observed with 7,8-benzoflavone. Piperine caused non-competitive inhibition of hepatic microsomal AHH from the untreated and 3-methylcholanthrene-treated rats with a K_i of 30 μM which was close to the apparent K_m of AHH observed in the controls. Similarly, the kinetics of inhibition of ethylmorphine-N-demethylase from control rat liver microsomes exhibited

non-competitive inhibition with a K_m of 0.8 mM and K_i of 35 μM. These studies demonstrated that piperine is a nonspecific inhibitor of drug metabolism which shows little discrimination between different cytochrome P_{450} forms. Singh *et al,* (1986) further demonstrated that piperine modifies the rate of glucuronidation by lowering the endogeneous UDP-glucuronic acid content and also by inhibiting the glucuronyl transferase activity.

Conclusion

Thus, the current observation of *in vitro* inhibition of the activities of hepatic microsomal aryl hydroxylase, N-demethylase and glucuronyl transferase by piperine is consistent with the earlier observation of Atal *et al*, (1985). It is also to be noted here that piperine has produced a negative effect on hepatic microsomal N-demethylase and glucuronyl transferase both *in vivo* and *in vitro*, while its effect on the aryl hydroxylase *in vivo* and *in vitro* was contrasting. The inhibitory effect of capsaicin and piperine *in vitro* on the electron transport particle cytochromeP_{450} is consistent with the reported type-I spectral change in rat hepatic microsomes produced by these to compounds (Miller *et al*, 1983; Atal *et al*, 1985). The inhibitory effect of piperine *in vitro* on hepatic cytochrome P_{450} is evidenced here to be far greater compared to that of capsaicin. This is the first report on the *in vitro* effect of these spice principles on hepatic cytochrome P_{450}. The same compounds however did not affect the cytochrome P_{450} or b_5 when fed continuously in the diet. Yet another interesting observation of the current study is the mild inhibitory effect of piperine on NADH-cytochrome C reductase *in vitro*, while the compound had no similar effect on the activity of NADPH-cytochrome C reductase.

GENERAL SUMMARY

1. Studies were made to examine the loss of curcumin, capsaicin and piperine, the active principles of turmeric, red pepper and black pepper respectively as a result of subjecting the spices to domestic cooking process. This involved heat treatment of each of these spices by (i) boiling for 10 min, (ii) boiling for 20 min and (iii) pressure cooking for 10 min.

2. Curcumin loss from heat processing of turmeric was 27 to 53%, with maximum loss in pressure cooking for 10 min. Curcumin loss from turmeric was similar even in presence of red gram. In presence of tamarind, the loss of Curcumin from turmeric was 12-30%.

3. Capsaicin losses from red pepper were in the range 18 – 36% with maximum loss being observed in pressure cooking. Presence of either red gram or tamarind or both did not influence the loss of Capsaicin.

4. Piperine losses from black pepper were in the range 16 – 34% with maximum loss being observed in pressure cooking. The loss was somewhat lower in presence of red gram.

5. The results of this investigation indicated diminished availability of spice active principles from cooked foods, where the food ingredients would have been subjected to either boiling or pressure cooking for few minutes.

6. In the present study, we have attempted to understand the nature of altered / degraded compounds formed from spice active principles – curcumin, capsaicin and piperine as a result of heat treatment. Attempts were also made to

structurally characterize the degradation products which are formed when curcumin / capsaicin / piperine was subjected to heat treatment.

7. Based on HPLC, LC-MS and NMR spectroscopy, three of the degraded compounds of curcumin were characterized as ferulic acid, vanillin and vanillic acid; while a few others could not be characterized. The UV absorption, proton NMR spectra and LC – MS/MS data of the three identified compounds matched very well with those of respective standards. Our studies confirm the vulnerability of the diketone bridge of curcumin molecule to heat. In addition, formation of vanillic acid and vanillin indicates that the molecule is sensitive to heat at the first carbon atom of the alkyl chain which is connecting the two benzene rings.

8. Although capsaicin and piperine were understood to undergo degradation as a result of heat treatment, the compounds derived from these respective molecules could not be structurally characterized due to experimental limitations.

9. A comparative evaluation of the absorbability of three structurally similar and physiologically active spice principles in an *in vitro* system consisting of everted rat intestinal sacs was made.

10. When everted sacs of rat intestines were incubated with 50-1000 μg of curcumin in 10 ml incubation medium, absorption of the spice principle was maximum at 100 μg concentration. The amount of absorbed curcumin present in the serosal fluid was negligible. This and the comparatively lower recovery of the original compound suggested that curcumin to some extent undergoes a modification during absorption.

11. For similar concentrations of added piperine, about 44 - 63 % of piperine disappeared from the mucosal side. Absorption of piperine which was maximum

at 800 μg per 10 ml was about 63%. The absolute amounts of piperine absorbed in this *in vitro* system exceeded the amounts of curcumin.

12. The absorbed piperine could be traced in both the serosal fluid and in the intestinal tissue, indicating that piperine did not undergo any metabolic change during the process of absorption. 7 - 12 % of the absorbed piperine was found in the serosal fluid.

13. When everted sacs of rat intestines were incubated with 10-500 μg of capsaicin, a maximum of 82-88 % absorption could be seen in the lower concentrations, and the amount of absorbed capsaicin did not proportionately increase at higher concentrations. A relatively higher percentage of the absorbed capsaicin could be seen in the serosal fluid as compared to curcumin or piperine.

14. When these spice active principles were associated with mixed micelles, their *in vitro* intestinal absorption was relatively higher. Curcumin absorption in everted intestinal sac increased from 48.7 to 56.1% when the same was present in micelles. In the case of capsaicin and piperine, increase in absorption was 27.8% to 44.4% and 43.4% to 57.4% respectively, when they were present in micelles as compared to its native form.

15. Tissue distribution and elimination of three structurally similar bioactive spice principles - curcumin, capsaicin and piperine following their oral administration was examined in rats.

16. Distribution in blood, liver, kidney and intestine in 1 - 24 h was 1.24 – 24.4 % of administered dose for capsaicin, 3.1-10.8 % for piperine, and 3.88 – 48.3 % for curcumin.

17. Maximum distribution of 24.4 % of administered capsaicin was seen at 1 h, while the same was not detectable after 4 days. Absorption of capsaicin was about 94% and very rapid relative to other two compounds.

18. A maximum of 10.8 % of administered piperine was seen in tissues at 6 h. Absorption of the administered piperine was about 96%.

19. Curcumin concentration was maximum in the intestine at 1h; maximum in blood at 6 h and remained at significantly higher level even at 24 h. About 63.5 % of the curcumin dose was absorbed.

20. Only a small portion of the administered dose of capsaicin (< 0.1%) and curcumin (0.173 %) was excreted in urine, whereas piperine was not detectable in urine.

21. Enhanced bioavailability of curcumin was evidenced when the same was orally administered concomitant with piperine. Intestinal absorption of curcumin was relatively higher, and it stayed significantly longer in the body tissues.

22. Intact curcumin was detected in brain at 24, 48 and 96 h with a maximum at 48 h.

23. Piperine, the bioactive alkaloid compound of black pepper exhibits a wide range of beneficial physiological and pharmacological activities. Being essentially water-insoluble, piperine is presumed to be assisted by serum albumin for its transport in blood. In the present investigation, interaction of the spice principle – piperine with human serum albumin (HSA) was been examined employing spectroscopic measurements – fluorescence and circular dichroism.

24. Binding of piperine to serum albumin was examined by employing steady state and time resolved fluorescence techniques. Binding constant for the interaction

of piperine with human serum albumin was observed to be 0.5 X 10^5 M^{-1} having stoichiometry of 1:1.

25. The binding constant was invariant with temperature in the range of 17^0C to 47^0C. At 27^0C, the van't Hoff enthalpy ΔH° was zero. ΔS° and ΔG° were found to be 21.4 cal.$mol^{-1}K^{-1}$ and – 6.42 kcal.mol^{-1}. The binding constant increased with the increase of ionic strength from 0.1 M to 1.0 M of sodium chloride. The decrease of Stern-Volmer constant with increase of temperature suggested that the fluorescence quenching is static in nature.

26. Piperine fluorescence showed a blue shift upon binding to serum albumin, which reverted with the addition of ligands such as triode benzoic acid and hemin.

27. The distance between piperine and tryptophan residue of albumin after binding was found to be 2.79 nm by FRET calculations.

28. The time resolved fluorescence study with BSA revealed that the amplitude of longer lifetime component decreased significantly with the increase in concentration of piperine.

29. The steady state and time resolved fluorescence measurements suggest the binding of piperine to the sub-domain IB of serum albumin. These observations are significant in understanding of the transport of piperine in blood under physiological conditions.

30. Capsaicin, the bioactive alkaloid compound of red pepper exhibits a wide range of beneficial physiological and pharmacological activities. Being essentially water-insoluble, capsaicin is presumed to be assisted by serum albumin for its transport in blood. In the present investigation, interaction of capsaicin with HSA was also

examined employing spectroscopic measurements – fluorescence, size exclusion chromatography and NMR.

31. Fluorescence measurement, Size exclusion chromatography and NMR suggested the binding of capsaicin with HSA. No further information on the binding of capsaicin to HSA could be drawn due to limitations such as overlapping fluorescence property of capsaicin with that of HSA, limited aqueous solubility of capsaicin, and lower molar absorption coefficient of capsaicin.

32. The effect of dietary supplementation of spice active principles – curcumin (0.2%), capsaicin (0.015%) and piperine (0.02%) on the activities of liver drug metabolizing enzyme system was examined in experimental rats.

33. All the three dietary spice principles significantly stimulated the activity of aryl hydroxylase. A synergistic action of dietary curcumin and capsaicin with respect to stimulating the activity of aryl hydroxylase was also evidenced when fed in combination.

34. The activity of N-demethylase while essentially remained unaffected by dietary curcumin, capsaicin or their combination, was significantly lowered as a result of piperine feeding.

35. UDP-glucuronyl transferase activity was decreased by dietary piperine and combination of curcumin and capsaicin. NADPH-Cytochrome C reductase activity was significantly decreased by dietary piperine.

36. The levels of hepatic microsomal cytochrome P_{450} and cytochrome b_5 were not influenced by any of the dietary spice active principles.

37. These spice active principles were also examined for their possible *in vitro* influence on the components of hepatic drug metabolizing enzyme system in rat liver microsomal preparation.

38. Piperine significantly decreased the activity of liver microsomal aryl hydroxylase activity when included in the assay medium at 1×10^{-6} M, 1×10^{-5} M and 1×10^{-4} M level. Lowered activity of N-demethylase was observed in presence of capsaicin or piperine at 1×10^{-6}M in the assay medium.

39. Hepatic microsomal glucuronyl transferase activity was significantly decreased *in vitro* by addition of capsaicin or piperine. Capsaicin and piperine brought about significant decrease in liver microsomal cytochrome P_{450} when included at 1×10^{-6} M and 1×10^{-5} M level, the effect being much higher in the case of piperine.

40. The results suggested that while the three tested spice principles have considerable similarity in structure, piperine is exceptional in its influence on liver drug metabolizing enzyme system. The study also indicated that a combination of curcumin and capsaicin does not produce any significant additive effect on liver drug metabolizing enzyme system.

41. Thus, the present investigation has provided significant information on the bioavailability of bioactive spice principles – curcumin, capsaicin and piperine of turmeric, red pepper and black pepper, respectively, in terms of losses encountered during domestic cooking, their intestinal absorbability, transport in blood, tissue distribution and elimination. It was envisaged in this investigation that considerable differences do exist among these three structurally related bioactive compounds with regard to their bioavailability.

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