DISPOSITION AND METABOLISM OF THE CANCER
CHEMOPREVENTIVE AGENT CURCUMIN IN RODENTS
AND HUMANS

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by

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myristate-13 acetate; RP, reversed phase; SD, standard deviation; SULT, sulphotransferase; TBST, tris buffered saline & tween; TCA, tricarboxylic acid; THC, tetrahydrocureumin TNFα, tumour necrosis factor alpha; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; UDPGA, uridine 5'-diphosphoglucuronic acid; UGTs, UDP-glucuronosyltransferases.
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Abstract: Disposition and Metabolism of the Cancer Chemopreventive Agent Curcumin in Rodents and Humans

Christopher Ireson

Curcumin, the major yellow pigment in the herb turmeric, has been shown to possess cancer chemopreventive activity in rodents. The bioavailability of curcumin in rodents is thought to be poor, but concentrations of $10^{-5}$-10$^{-4}$ M curcumin are required for biological activity. The role of curcumin metabolites in the biological efficacy of curcumin is not clear. In order to improve our understanding of pharmacokinetic issues which may impinge on the pharmacology of curcumin, its disposition and metabolism was studied in three different settings: i) in subcellular fractions of intestine and liver from rodents and humans, ii) in intact hepatocytes isolated from rats and humans, and iii) in vivo in rats which had received curcumin. Cytosol from intestine or liver metabolised curcumin to curcumin sulphate and hexahydrocurcumin, microsomes biotransformed curcumin to curcumin glucuronide, hepatocytes metabolised curcumin to hexahydrocurcumin and hexahydrocurcuminol. Metabolic reduction of curcumin in human intestine was 18 times more abundant than that in rat intestine. When curcumin was administered by gavage, curcumin, curcumin sulphate and curcumin glucuronide were detected in plasma. When rats received curcumin as a dietary constituent, only minute levels of curcumin were measured in the plasma. In a pilot study of a standardised turmeric formulation in colorectal cancer patients, curcuminoids could not be identified in the blood after doses of up to 180 mg curcuminoids per day. Curcumin sulphate and hexahydrocurcumin were generated from curcumin by isolated sulphotransferase 1A1/1A3 and equine alcohol dehydrogenase, respectively. The results demonstrate that curcumin is rapidly metabolised by human and rat hepatic and intestinal tissues, which may explain, at least in part, its poor bioavailability. In conclusion, the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent.
1 Introduction
1.1 Carcinogenesis and its prevention

Cancer is the second leading cause of death in most developed countries including the UK [La Vecchia et al., 1998] and its incidence is increasing in most countries. In 1999, there were approximately 150,000 cancer related deaths in the United Kingdom [Swerdlow et al., 2001]. Invasive tumours develop over many years via a multi-stage pathway [Hanahan and Weinberg, 2000], yet tumours are not usually diagnosed until a relatively late stage of the disease process. Traditional chemotherapy involves the use of drugs that are cytotoxic to cancer cells. The disadvantage of this approach is that these cytotoxic agents often kill normal cells as well as tumour cells [Sporn and Suh, 2000]. Interfering with carcinogenesis before tumours have been established offers an alternative strategy of cancer management. Chemoprevention was defined by Sporn as “the use of specific natural or synthetic agents to reverse, suppress or prevent the carcinogenic process to invasive cancer” [Sporn, 1976]. During the last twenty-five years, polyphenolic compounds have emerged as an important group of potential cancer chemopreventive agents that are available in the diet. Examples of compounds currently under evaluation for chemopreventive activity include the soy constituent, genistein [Messina et al., 1994]; epigallocatechin gallate (EGCG), which is found in green tea; and curcumin, the principal ingredient in the herb turmeric. The efficacy and safety of these dietary agents needs to be elucidated thoroughly in humans before these agents can be given to the general population to prevent cancer; analogous to the way in which fluoride is added to drinking water in order to prevent the onset of dental decay [Anon, 1999].

The development of a malignant tumour is a consequence of a complex interaction between exogenous (e.g. environmental) and endogenous (e.g. genetic) factors [Hanahan and
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Weinberg, 2000]. The carcinogenic process has traditionally been divided into three distinct phases: initiation, promotion and progression (see Figure 1.1)[Foulds, 1969], which may occupy a large portion of the lifespan of the individual. Chemopreventive agents may act at one or more of the stages of carcinogenesis. Essentially the initiation stage consists of irreversible DNA damage, which may be inherited or acquired. The clonal expansion of cells, which have undergone a selection advantage, is referred to as promotion. This step in the carcinogenic process is reversible and consequently is a particularly attractive target for chemoprevention. Progression is the term used to describe the continued evolution of mutated cells. A chemopreventive agent that affects the initiation stage is referred to as a tumour blocking agent [Watterneg, 1985]. Tumour suppressing agents are those compounds that affect the promotion/progression stages of carcinogenesis [Watterneg, 1985]. The carcinogenic process is now thought to be more complex than the three-stage process described here would imply [Gescher et al., 2001]. Carcinogenesis is thought to result from multiple genetic mutations [Weinstein et al., 1995] in addition to changes in the profile of molecules that regulate cell proliferation. However the model described here allows chemopreventive agents to be divided into those that block initiation and those that suppress proliferation of cells in which carcinogenesis has been initiated.
Figure 1.1. Hypothetical stages of the carcinogenic process [Gescher et al., 1998]. The X represents damaged DNA.
1.1.1 Pre-clinical and clinical development of chemopreventive agents

There are two main stages in the preclinical development of chemopreventive agents. The first stage consists of elucidating the mechanism of action of the compound in vitro. Secondly, the agent is tested for biological efficacy and safety in vivo. There is considerable debate as to whether purified agents or a mixture of dietary agents should be used in chemoprevention studies. There are several advantages of using a mixture of dietary chemopreventive agents. Firstly, the food matrix contains a plethora of potential chemopreventive agents that may have a synergistic effect. Indeed, results of a recent study demonstrated that the oxygen radical scavenging ability of an apple extract was greater than would be anticipated on the basis of its vitamin C content alone [Eberhardt et al., 2000]. Secondly, it is likely that the food matrix containing the chemopreventive agent would have been consumed by large numbers of individuals for relatively long periods of time. Consequently toxic side effects are more likely to be associated with the single purified agent than with the mixture of agents present in foodstuffs. At the Division of Cancer Prevention of the National Cancer Institute (NCI), United States (US), both single agents and food extracts are compared in vitro for biological efficacy. If the chemopreventive agents from both sources are found to have similar efficacy, the mixture of agents is used. If the isolated single agent has greater efficacy then this is selected for in vivo testing. Agents are administered to animals at risk of cancer development in order to obtain information about efficacy, optimum dose and therapeutic index. The in vitro and in vivo preclinical work is a vital part of the development of chemopreventive agents. The importance of the pre-clinical work is emphasised by the negative outcome of two large clinical trials involving β-carotene [The alpha Tocopherol, 1994; Ommen et al., 1996a]. The trials, involving approximately 50,000
individuals, were based on epidemiological evidence, which suggested that diets rich in β-carotene could potentially decrease the risk of developing lung cancer. Subgroup analysis of the individuals participating in these trials revealed that consumption of β-carotene actually increased the risk of developing lung cancer in individuals who continued to smoke 20 cigarettes or more per day or those who were in the highest quartile of alcohol consumption [Albanes et al., 1996; Ommen et al., 1996b].

Animal models cannot be considered as a substitute for humans in determining the efficacy of chemopreventive agents. There are inter-species differences in drug metabolism and disposition and tumour development. Clinical trials are necessary in order to determine the dose, efficacy, safety and dosing schedule. Hong et al. was the first to demonstrate that 13-cis-retinoic acid could reduce the incidence of oral leucoplakia [Hong et al., 1986] and head and neck cancer [Hong et al., 1990]. Recently, a 49% decrease in the risk of developing breast cancer was associated with tamoxifen treatment [Fisher et al., 1998]. Further clinical studies are underway to determine whether raloxifene might cause a reduction in breast cancer risk without the increase in endometrial cancer risk associated with tamoxifen.

Trials of chemopreventive agents are generally divided into three phases [Kelloff et al., 1995]. In phase I trials the pharmacokinetics and toxicity of the agent are investigated in 15-40 individuals. In addition surrogate biomarkers, which indicate the biological efficacy of the agent, should be identified. The aim of a phase II trial is to investigate the biological efficacy of the agent using the dose and surrogate biomarkers identified during the phase I trial. The trial should also contain a placebo group of individuals that do not receive the agent. The trial should be double blind, which means that neither the doctor nor the participant are aware of whether the agent or a placebo is being taken. If phase I and II trials of an agent are
successful then the agent will proceed to phase III investigations. Phase III trials involve large numbers of individuals (frequently in the order of 50,000) and are consequently expensive. Intermediate endpoints of efficacy include monitoring surrogate biomarkers identified during phase I and II trials and also new biomarkers. The long-term endpoint is the incidence of primary tumours.

1.2 Colorectal cancer

Cancer of the colon and rectum accounts for 18,000 deaths in the UK each year and is the second commonest cancer in most developed countries [Franks et al., 1997]. The risk of developing colorectal cancer is associated with both genetic predisposition and the effect of environmental factors such as the diet. These risk factors appear to include diets that are low in vegetables and fibre and high in fat, red meat, alcohol and cigarette smoking. Two inherited genetic syndromes have been associated with colorectal cancer: familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). The remaining cases are termed sporadic colorectal cancer and these account for the majority of colorectal cancer cases. Vogelstein proposed a multistep model for the genetic events that lead to sporadic colorectal cancer [Vogelstein et al., 1988]. Colorectal cancer occurs mainly during the sixth and seventh decade of life and this is consistent with the theory that a cancer cell must develop four or five genetic defects in order to undergo malignant transformation. These defects include mutational activation of oncogenes and inactivation of tumour-suppressor genes.
1.3 Curcumin

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major yellow pigment extracted from turmeric, the dried ground rhizome of the herb *Curcuma Longa*. The spice turmeric is extensively used to impart colour and flavour to food and is a principal ingredient in curry powder. Turmeric has also been used medicinally in the Asian subcontinent as a treatment for a diverse range of conditions including wounds, haemorrhage, inflammation, menstrual difficulties, chest pain and cancer [Ammon and Wahl, 1991]. In India normal dietary intake of turmeric has been found to range from 0.1 g to 3.8 g/adult/day [Shankar et al., 1979]. Turmeric contains between 2 and 8% curcumin depending on the source used [Sambahiah et al., 1982]. Interestingly, undeveloped countries have a lower incidence of colorectal cancer than developed countries [Greenlee et al., 2000], which may at least in part, be attributable to the contribution of dietary factors. There is preliminary evidence for the chemopreventive efficacy of curcumin in humans. In one clinical trial, the urinary mutagen load was lowered in chronic smokers who were given turmeric at a dose of 1.5 g/day for 30 days [Polasa et al., 1992]. Curcumin has also been shown to reduce serum cholesterol levels in healthy individuals receiving 500 mg curcumin for 7 days [Soni and Kuttan, 1992].

1.3.1 Chemistry and stability of curcumin

The structure of the polyphenolic compound curcumin is shown in Figure 1.2. The keto-enolate equilibrium in the heptadienone moiety is pH dependent. In neutral and acidic conditions (pH 3-7), the bis-keto form predominates, whereas under pH conditions of greater
than 8, the enolate form predominates [Jovanovic et al., 1999]. The position of the equilibrium can affect the antioxidant properties of curcumin, as discussed in section 1.3.3.
Figure 1.2. Tautomerism of curcumin under physiological conditions. Under acidic and neutral conditions the bis-keto form (A) predominates, whereas the enolate form predominates in conditions where the pH is greater than 8 (B).
The stability of curcumin in phosphate buffer at various pH values has been investigated [Wang et al., 1997]. When curcumin was incubated in 0.1 M phosphate buffer (pH 7.2) at 37°C, 90% of the agent decomposed within 30 min [Wang et al., 1997]. At constant pH and ionic strength the degradation rate of curcumin has been found to be similar in both phosphate and carbonate buffers [Tonnesen and Karlsen, 1985]. The concentration of buffer used did not affect the rate of curcumin degradation [Tonnesen and Karlsen, 1985]. The stability of curcumin was found to be increased at lower pH values: the half-life of curcumin in 0.1 M phosphate buffer at pH 3.0 was 118 min [Wang et al., 1997]. Consequently curcumin may be stable under the acidic conditions of the upper gastrointestinal tract. Curcumin has been found to be more stable in cell culture medium containing 10% foetal calf serum (FCS) and human blood than when it is maintained in phosphate buffer at an equivalent pH [Wang et al., 1997]. Indeed only 50% decomposition of curcumin was measured after incubation in human blood at 37°C for 8 hr. It is conceivable that binding of curcumin to plasma protein results in the increased stability of this agent in blood [Reddy et al., 1999]. Mass spectrometric analysis allowed the identification of the degradation products as vanillin, ferulic acid and feruloyl methane (see Figure 1.3) [Wang et al., 1997]. In order to investigate the stability of curcumin under common cooking conditions, it was incubated at 100°C for 15 min [Srinivasan et al., 1992]. During this 15 min incubation, there was 20-28% and 41-45% decomposition of curcumin at pH 5.1 and 6.1, respectively. The mechanism of curcumin degradation is unclear, although it is conceivable that it is via an oxidative mechanism, because the presence of antioxidants such as ascorbic acid, N-acetylcysteine or glutathione completely block degradation [Pan et al., 1999].
1.3.2 Chemopreventive efficacy of curcumin in vivo

The chemopreventive efficacy of curcumin has been demonstrated in vivo using chemical and genetic animal models of carcinogenesis. In the chemical model, carcinogenesis was induced by exposure of the animal to high doses of carcinogens, e.g. benzo[a]pyrene (B[a]P), 7,12-dimethylbenz[a]anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA) and azoxymethane (AOM). Topical application of curcumin to mouse skin was found to decrease the multiplicity of tumours previously initiated with TPA [Huang et al., 1988]. Several studies have shown that curcumin can protect against colon tumour formation in the rat [Rao et al., 1995a; Rao et al., 1995b; Pereira et al., 1996]. In subsequent studies, tumours were initiated in mouse forestomach and duodenum with B[a]P and N-ethyl-N-nitro-N-nitroguanidine respectively. Curcumin was found to inhibit tumour formation in both these
tissues. When γ-radiation was used to initiate carcinogenesis in the mammary gland of the rat, curcumin reduced tumour formation at both the initiation [Inano et al., 2000] and the promotion/progression [Inano et al., 1999] stages of carcinogenesis. Similarly curcumin (0.2%) in the diet inhibited the development of AOM induced colonic tumours irrespective of whether the compound was administered at the initiation [Huang et al., 1994] or the progression [Kawmori et al., 1999] stage of the disease process. The multiple intestinal neoplasia (MIN) mouse is an example of a genetic model of colon carcinogenesis. The heterozygous MIN mouse has an autosomal mutation in the adenomatous polyposis coli gene (APC) that results in the spontaneous formation of intestinal adenomas. The mutation in the mouse is homologous to human germ-line and somatic APC mutations. When curcumin was administered to heterozygous MIN mice as a dietary constituent at levels of 0.2 and 0.5%, it reduced the number of adenomas formed by by 39 and 40% respectively, compared to the mice that received the control diet [Perkins et al., 2002].

1.3.3 Mechanisms of action of curcumin

Experiments with cultured cell lines have elucidated potential mechanisms by which curcumin may prevent cancer. Curcumin has been shown to act at both the initiation [Inano et al., 2000; Huang et al., 1994] and promotion/progression [Inano et al., 1999; Kawmori et al., 1999] stages of carcinogenesis in vivo.

Curcumin may act as a tumour blocking agent by affecting the activities of drug metabolising enzymes. Phase I drug metabolising enzymes, such as cytochromes P450 (CYP) 1A1 and 1A2 have a role in activation of carcinogens [Timbrell, 1991]. Curcumin has been demonstrated to be an inhibitor of CYP1A1/1A2 in rat liver microsomes [Oetari et al., 1996].
CYP1A1 and 1A2 are known to activate procarcinogens to their ultimate carcinogens, e.g. the metabolism of the hepatocarcinogen aflatoxin B\(_1\) (AFB\(_1\)) to the ultimate electrophilic carcinogen AFB\(_1\)-8,9-epoxide [Wogan, 1973]. Similarly the polycyclic hydrocarbon B[a]P, a procarcinogen found in cigarette smoke is metabolised by CYP1A1 and 1A2 to form the ultimate carcinogen B[a]P 7,8-dihydrodiol,9,10-oxide (BPDE) [Timbrell, 1991]. Therefore curcumin may exert its chemopreventive effect by reducing the formation of ultimate carcinogens, which can potentially bind to DNA and form adducts. Indeed, in a reconstituted microsomal system, modulation of cytochromes P450 activity by pre-incubation with curcumin resulted in decreased aflatoxin-B\(_1\)-DNA adduct formation [Firozi et al., 1996]. Singh et al. demonstrated that 2% curcumin in the diet inhibited B[a]P induced forestomach cancer in mice and decreased levels of hepatic CYP1A1 [Singh et al., 1998]. Phase II enzymes, e.g. glutathione-S-transferases (GSTs) are primarily associated with the detoxification of carcinogenic compounds. Curcumin admixed in the diet at a level of 2% was found to increase hepatic GSTs in the rat [Sharma et al., 2001a; Singh et al., 1998]. In contrast, Oetari et al. found that curcumin inhibited microsomal GST activity in vitro [Oetari et al., 1996].

Curcumin has also been shown to affect the promotion/progression stage of carcinogenesis in vivo (see section 1.3.2). The inappropriate regulation of signalling pathways such as those that involve nuclear factor-kappa B (NF-κB) is associated with acute inflammation and cancer [Manson et al., 2000]. Modulation of cell signalling pathways by curcumin may affect the transcription of key genes involved in the promotion/progression stages of carcinogenesis. Curcumin has been shown to inhibit activation of c-Jun/AP-1. These are transcription factors that are involved in the formation of tumours in mouse fibroblast cells induced with phorbol 12-myristate-13 acetate (PMA) [Huang et al., 1991]. NF-κB is a cytosolic transcription factor...
that is rendered inactive by the NF-κB sequestering protein, IκB (IκB) [Simeonidis et al., 1996]. The NF-κB signalling pathway can be activated by stimulation with bacterial lipopolysaccharide (LPS) or tumour necrosis factor (TNFα). Following stimulation of the NF-κB signalling pathway, the IκB group is phosphorylated and targeted for degradation, allowing the activated transcription factor, NF-κB, to migrate to the nucleus and exert its downstream effects. Curcumin has been shown to inhibit activation of the NF-κB pathway in myeloid cells [Singh and Aggarwal, 1995] and in colon cells [Plummer et al., 1999]. Furthermore, these researchers demonstrated that curcumin prevents phosphorylation of IκB by inhibition of I kappa B kinases (IKKs) (see Figure 1.4) [Plummer et al., 1999]. NF-κB regulates a plethora of genes associated with inflammation including cyclooygenase-2 (COX-2). COX-1 and 2 are key regulatory isoenzymes in the metabolism of arachidonic acid to prostaglandins. Elevated levels of prostaglandins have been associated with cancer of the breast [Bennett et al., 1977], lung [Bennett et al., 1982], colon [Rigas et al., 1993] and neck [Bennett et al., 1980]. COX-1 is constitutively expressed in most tissues where it mediates the synthesis of prostaglandins that are required for normal physiological functions, for example platelet aggregation [Arita et al., 1989]. In contrast, increased levels of inducible COX-2 have been associated with many pathological states including human cancers of the colon [Eberhart et al., 1994; Kargman et al., 1995; Sano et al., 1995], stomach [Risitimak et al., 1997] and breast [Parrett et al., 1997]. Curcumin has been shown to inhibit expression of COX-2 in several colon cancer cell lines [Zhang et al., 1999]. Curcumin also directly inhibits the activity of the COX-2 enzyme [Rao et al., 1995b; Zhang et al., 1999]. Activation of the NF-κB pathway has also been shown to inhibit apoptosis [Beg and Baltimore, 1996]. Therefore inhibition of this pathway by curcumin may help to maintain normal levels of cellular apoptosis. Indeed the ability of curcumin to induce apoptosis has been demonstrated
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in the promyelotic leukaemia HL-60 cells [Kuo et al., 1996] and several immortalised cell lines including human colon epithelial cells (HCEC) and the malignant colon cell line, HT-29 [Jiang et al., 1996]. The NF-κB pathway has also been shown to have a pivotal role in enhanced gene expression of inducible nitric oxide synthase (iNOS) in macrophages [Xie et al., 1994]. iNOS catalyses the endogenous formation of nitric oxide (NO). Increased NO expression has been associated with cancer of the breast [Thomsen et al., 1995] and central nervous system [Cobbs et al., 1995]. Curcumin has been shown to inhibit induction of iNOS in activated macrophages [Brouet and Oshima, 1995]. Furthermore the inhibition of iNOS expression by curcumin has been shown to be mediated by inhibition of IKK activity and NF-κB activation [Pan et al., 2000].

Curcumin has also been demonstrated to be an inhibitor of phosphorylase kinase, a key regulatory enzyme in the metabolism of glycogen [Reddy and Aggarwal, 1994].

Reactive oxygen species (ROS) are generated both by exogenous sources, (e.g. cigarette smoke) and endogenously (e.g. phagocytic cells involved with the immune response). These ROS can potentially cause damage to DNA, protein and lipids. There are several lines of evidence that demonstrate that ROS, such as singlet oxygen, peroxy radicals, superoxide anion and hydroxy radicals are involved in carcinogenesis [Kensler et al., 1992]. Firstly, oxygen radical generating systems, e.g. superoxide generation via xanthine oxidase, show activities in vitro similar to those of known tumour promoters. These activities include increasing transformation frequencies of fibroblasts and keratinocytes and increasing transcription of genes associated with early steps in cell proliferation including c-fos, c-myc, c-jun and ornithine decarboxylase. Secondly, inflammatory cells produce a range of reactive oxygen species and there is evidence associating inflammation with cancers in various tissues.
including stomach, oesophagus, colon/rectum and bladder. Thirdly, tumour promoters stimulate the endogenous production of oxygen radicals in inflammatory cells and keratinocytes. Fourthly, tumour promoters inhibit endogenous activities which protect against oxidative damage, such as those of glutathione peroxidase, catalases and superoxide dismutase. Finally, free radical-generating agents such as benzoyl epoxide and butylated hydroxytoluene hydroperoxide are tumour promoters in mouse skin. Joe and Lokesh demonstrated the ability of curcumin to inhibit generation of superoxide, hydrogen peroxide and nitrite radicals in rat macrophages, both in vitro and in vivo [Joe and Lokesh, 1994]. Jovanovic et al. proposed a mechanism for the antioxidant capacity of curcumin [Jovanovic et al., 1999]. It was postulated that in acidic and neutral conditions, donation of a hydrogen atom from the aliphatic chain of the molecule occurs. The donation of the hydrogen atom is made possible by delocalisation of the non-bonding electrons on the adjacent oxygen atoms [Jovanovic et al., 1999]. In alkaline conditions, the enolate form of curcumin predominates (see Figure 1.2) and electron donation of the phenolic group becomes the main antioxidant site in the molecule. In contrast Barclay et al. showed that synthetic non-phenolic curcuminoids do not have any antioxidant activity and therefore curcumin acts as a "classical antioxidant" by donation of H atoms from its phenolic ring [Barclay et al., 2000].

Clearly the anti-mutagenic and anti-carcinogenic effects of curcumin result from numerous mechanistic processes and it is difficult to postulate which of these, if any, are of predominant importance.
Figure 1.4. Stimulation of NF-κB activation by tumour necrosis factor alpha (TNF-α). Upon activation of IKK, IκB is phosphorylated and targeted for degradation. The activated transcription factor, NF-κB, translocates to the nucleus whereby it can modulate the expression of genes involved with inflammation, for example COX-2. Curcumin is thought to prevent phosphorylation of IκB by inhibiting the activity of the IKKs.
1.3.4 Absorption, distribution, metabolism and excretion of curcumin

a) Absorption

Curcumin appears to be poorly absorbed from the gastrointestinal tract in rodents [Wahlstrom and Blenhow, 1978; Pan et al., 1999]. When a dose of 1,000 mg/kg curcumin as a suspension in arachis oil was administered by intragastric (i.g.) intubation to Sprague-Dawley rats, low levels of parent compound were detected in the plasma [Wahlstrom and Blenow, 1978]. Three hours following dosing with curcumin, the compound could only be detected in the plasma of 25% of the animals. The concentration of curcumin in the plasma of humans [Shoba et al., 1998], mice [Pan et al., 1999] and rats [Asai and Miyazawa, 2000] reached their highest values between 0.5 and 1 hr after dosing. It is possible that absorption, as reflected by curcumin plasma levels, may be affected by the vehicle and the species, strain or sex of animal used (see Table 1.1). If the dose of 2,000 mg/kg administered to rats shown in Table 1.1 were considered necessary for human dosage, a 70 kg individual would have to consume 140 g of curcumin. Freireich et al. investigated the maximum tolerated dose (MTD) of anticancer agents in humans, mouse, rat, hamster, dog and monkey [Freireich et al., 1966]. It was observed that the most accurate prediction of MTD in humans could be determined using the surface area (mg/m²) as opposed to weight (mg/kg). The dose according to surface area (mg/m²) can be calculated by multiplying the dose measured in mg/kg by a constant (Km). Km is the appropriate factor for inter-conversion of mg/kg and mg/m² for each species. The doses according to surface area were calculated for each of the absorption studies using the appropriate factors and added to Table 1.1.
In a phase I clinical study, the average serum curcumin concentration was determined to be 1.75 μM (0.64 μg/ml) following oral consumption of 8,000 mg curcumin/individual in capsule formulation [Cheng et al., 1999; Cheng et al., 2001]. Ravindranath et al. investigated the absorption of curcumin in male albino Wistar rats after administration of approximately 2,000 mg/kg curcumin [Ravindranath and Chandrasekhara, 1980] and 40, 320 and 2,000 mg/kg of tritiated curcumin [Ravindranath and Chandrasekhara, 1982]. The absorption of curcumin was determined to be approximately 60% regardless of the dose given. This value may represent an over-estimation of curcumin absorption, as it was calculated by determination of the amount of unchanged curcumin excreted in the faeces.
## Table 1.1. Summary of maximum levels of curcumin in the plasma following a single i.g. dose of curcumin. The plasma concentrations shown in the table were calculated using HPLC. The curcumin plasma concentrations were determined by the following investigators: *\[Shoba et al., 1998\], [Asai and Miyazawa, 2000], [Pan et al., 1999] and [Cheng et al., 1999]*

It is conceivable that the poor bioavailability of curcumin is a result of avid intestinal and/or hepatic metabolism. In an attempt to increase the bioavailability of curcumin in rats and humans, a study was conducted in which curcumin was co-administered with piperine (l-piperoylpiperidine), a constituent of black pepper [Shoba et al., 1998]. Piperine has previously been reported to increase the bioavailability of compounds by inhibition of glucuronidation in the liver [Atal et al., 1985] and intestine [Singh et al., 1985]. Shoba et al. showed that higher plasma levels of parent compound were attained in humans and rats when

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sex</th>
<th>Dosing Medium/Vehicle</th>
<th>Dose (mg/kg)</th>
<th>Dose (mg/m²)</th>
<th>Plasma Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>Male &amp; Female</td>
<td>Aqueous suspension</td>
<td>2,000</td>
<td>12,000</td>
<td>1.0ₐ</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>Male</td>
<td>1% sodium cholate</td>
<td>100</td>
<td>600</td>
<td>0.2ₐ</td>
</tr>
<tr>
<td>Mouse</td>
<td>BALB/c</td>
<td>Female</td>
<td>2.5% carboxymethyl cellulose</td>
<td>1,000</td>
<td>3,000</td>
<td>0.2₂₉</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td>Male</td>
<td>Capsule</td>
<td>8,000</td>
<td>320,000</td>
<td>0.6₄ₙ</td>
</tr>
</tbody>
</table>
curcumin was administered in conjunction with piperine than when curcumin was given alone [Shoba et al., 1998]. The authors concluded that the increased plasma levels of curcumin were due to decreased glucuronidation of parent compound.

b) Distribution

Ravindranath et al. studied the distribution of curcumin in male albino Wistar rats following oral dosing of approximately 2,000 mg/kg curcumin [Ravindranath and Chandrasekhar, 1980] and 40, 320 and 2,000 mg/kg tritiated curcumin [Ravindranath and Chandrasekhar, 1982]. Following administration of radiolabelled curcumin, the levels of total radioactivity in blood, liver and kidney was found to be in the range of 9.6-14%, 5.1-6.9% and 14.7-23.3% of the administered dose respectively [Ravindranath and Chandrasekhar, 1982]. Only trace levels of curcumin could be detected in portal blood, liver and kidney between 15 min and 24 hr following oral administration of cold curcumin [Ravindranath and Chandrasekhar, 1980]. The majority of the curcumin dose accumulated in the gastrointestinal tract. Thirty minutes following administration of curcumin, 90% of the dose given could be accounted for in the stomach and small intestine. After seven hr, the maximum levels of curcumin were measurable in the caecum and the large intestine, with only small amounts of the agent detectable in the small intestine and stomach. After 24 hr, only 1% of the dose given remained in the stomach. The distribution of curcumin in mice 1 hr after an intraperitoneal (i.p.) dose of 100 mg/kg has also been investigated [Pan et al., 1999]. The concentration of curcumin in the gastrointestinal tract (117 µg/g) was approximately six times greater than in the liver and spleen. Only trace levels of curcumin were detected in the plasma and brain.
c) Metabolism

The metabolites of curcumin were initially identified in the bile of male Sprague-Dawley rats [Holder et al., 1978]. Curcumin was administered to rats by the i.v. route at a dose of approximately 50 mg/kg. Bile was collected by cannulation of the bile ducts. Curcumin metabolites were identified by mass spectrometric analysis as glucuronide conjugates of tetrahydrocurcumin (THC) and hexahydrocurcumin (HHC) (see Figure 1.5) [Holder et al., 1978]. Traces of dihydroferulic acid and ferulic acid (see Figure 1.3) were also identified in the bile [Holder et al., 1978]. Ferulic acid has been identified as a degradation product of curcumin in phosphate buffer [Wang et al., 1997]. In recent studies, curcumin glucuronide and THC glucuronide were identified as plasma metabolites in mice following an i.p. dose of 100 mg/kg and i.g. dose of 1,000 mg/kg [Pan et al., 1999]. When 100 mg/kg curcumin was administered to male Sprague-Dawley rats, curcumin glucuronide and curcumin glucuronide sulphate were identified as plasma metabolites [Asai and Miyazawa, 2000].

The in vitro metabolism of curcumin has been studied in two systems. Firstly, curcumin was incubated with isolated rat hepatocytes and microsomes [Wahlstrom and Blenow, 1978], and 90% of substrate was removed from the media within 30 min. Secondly, Asai et al. investigated the ability of rat liver, kidney, intestinal mucosa homogenate and plasma to conjugate curcumin [Asai and Miyazawa, 2000]. The amount of curcumin sulphate and curcumin glucuronide generated by the tissue homogenates was calculated indirectly as an inference of curcumin disappearance. Consequently curcumin glucuronide and curcumin sulphate were not unambiguously identified as metabolic products of these tissues. UDP-glucuronosyl transferase (UDPGT) and sulphotransferase (SULT) activity was found in all tissue preparations assayed, with the exception of plasma. The highest SULT and UDPGT activity was measured in the liver homogenate.
Figure 1.5. Chemical structures of curcumin and its metabolites in mice, rodents and humans
d) Excretion

When a dose of 1,000 mg/kg curcumin was administered as an i.g. bolus to male and female *Sprague-Dawley* rats, only 0.001% of the dose was excreted in the bile [Wahlstrom and Blenow, 1978]. However when a dose of 20 mg/kg of tritiated curcumin was administered intravenously to male *Sprague-Dawley* rats, 85% of the radiolabelled was recovered in the bile within three hours [Holder *et al.*, 1978]. The radiolabelled compounds in the bile were identified as glucuronide conjugates of THC and HHC. Curcumin and its metabolites have not been detected in the urine following administration of a single oral dose of 2,000 mg/kg curcumin [Ravindranath and Chandrasekhara, 1980] and tritiated curcumin [Ravindranath and Chandrasekhara, 1982] to male albino *Wistar* rats. However Ravindranath *et al.* measured an increase in total urinary glucuronide and sulphate conjugates between 1 and 8 days after dosing with curcumin [Ravindranath and Chandrasekhara, 1980]. It was established that the faeces was the main route of excretion. When an oral dose of 2,000 mg/kg curcumin was administered to male albino *Wistar* rats, 40% of the agent was excreted unchanged in 72 hrs [Ravindranath and Chandrasekhara, 1980]. Wahlstrom *et al.* found that 65-80% of an i.g. dose of 1,000 mg/kg curcumin was excreted unchanged in the faeces [Wahlstrom and Blenow, 1978]. After administration of 40 and 320 mg/kg tritiated curcumin to male albino *Wistar* rats, 80-90% of the radiolabelled compound was excreted within 72 hrs [Ravindranath and Chandrasekhara, 1982]. At the higher dose of 2,000 mg/kg, the authors found that only 33% of the administered dose was excreted in the faeces within 72 hr. Holder *et al.* also found that 90% of an i.v. dose of 20 or 50 mg/kg tritiated curcumin was excreted within 3 hr [Holder *et al.*, 1978].
1.3.5 Toxicity of curcumin

Ultimately it is likely that individuals will be exposed to chemopreventive agents for prolonged periods of time. Therefore it is imperative that these compounds should have negligible toxic effects. Curcumin is perceived as being safe for human consumption following its extensive use in the Indian subcontinent as a dietary constituent and in herbal remedies. Doses of up to 5,000 mg/kg of curcumin have been administered to male and female Sprague Dawley rats without causing any significant changes in behaviour or relative organ weights [Wahlstrom and Blenow, 1978]. Furthermore, no toxicity was observed in rats, guinea pigs and monkeys when doses of up to 2,500 mg/kg turmeric were administered [Shankar et al., 1979]. In two clinical trials in India, toxicity was not observed in individuals receiving 400 mg curcumin capsules four times daily [Ammon and Wahl, 1991] or in volunteers treated with 500 mg curcumin daily for seven days [Soni and Kuttan, 1992]. Results from a phase I clinical trial also indicate a lack of side effects following oral doses of 8,000 mg daily [Cheng et al., 1999; Cheng et al., 2001].
1.4 Phase II metabolism of xenobiotic compounds

Phase II metabolic routes include acetylation, methylation, glucuronidation, sulphation and conjugation with glutathione or amino acids. Curcumin glucuronide was identified as a metabolite of curcumin in the plasma of mice [Pan et al., 1999] and rats [Asai and Miyazawa, 2000]. Many xenobiotic and endogenous compounds containing a phenolic moiety are metabolised by addition of a sulphate or glucuronide group. The sulphate and glucuronide metabolites are more water-soluble than their precursor molecules. Consequently these metabolic routes lead to an enhancement in the excretion of xenobiotic compounds from the body [Mulder et al., 1990].

1.4.1 Glucuronidation

Glucuronidation is a major pathway of drug metabolism in most mammalian species with the exception of the cat family [Burchell and Coughtrie, 1992]. The site of glucuronidation is usually a nucleophilic heteroatom (e.g. O, S or N). Consequently substrates for glucuronidation include aliphatic alcohols and phenols (which form O-glucuronide ethers), carboxylic acids (which form O-glucuronide esters) and aliphatic amines (which form N-glucuronides). Substrates for glucuronidation include xenobiotic compounds and endogenous compounds, e.g. bilirubin. Glucuronide conjugates are excreted either in the bile or urine and this depends primarily on the molecular weight of the compound. In the rat, conjugates with a molecular weight of less than 250 are predominantly excreted in the urine, whereas larger molecules are preferentially eliminated in the bile. The transfer of glucuronic acid from the cofactor, uridine 5'-diphosphoglucuronic acid (UDPGA), to the substrate is catalysed by a
family of microsomal UDP-glucuronosyl transferases (UGTs). These enzymes are located in the liver and other tissues, such as the lung [Aitio, 1976], small intestine [Dahl-Pustinen et al., 1989], skin [Coomes et al., 1983] and nasal mucosa [Lazard et al., 1991]. The cofactor required for these reactions, UDPGA, is generated from glucose-1-phosphate (see Figure 1.6).

UDPGA has an α configuration which means that it is stable to the attack of β-glucuronidase. However, glucuronide conjugates have a β configuration, because the nucleophilic heteroatom (e.g. O) group attacks UDPGA on the opposite side from the UDP moiety (see Figure 1.6). Consequently, β-glucuronides are susceptible to cleavage by β-glucuronidases, which are present in the intestinal microflora. The cleavage of the glucuronide group by intestinal β-glucuronidases can result in reabsorption of the original compound, a process known as enterohepatic circulation. This process effectively decreases the rate of elimination of xenobiotic compounds.

UGTs are located in the endoplasmic reticulum (ER) and the enzyme is orientated towards the lumenal side [Burchell and Coughtrie, 1989]. The water-soluble cofactor, UDPGA is synthesised in the cytosol and consequently has to be transferred into the ER, presumably via a transporter protein. In vitro work with microsomes generally involves use of a detergent, e.g. Triton X-100, which disrupts the microsomal membrane integrity and facilitates interaction between the cofactor and enzyme.
Figure 1.6. The synthesis of UDPGA from glucose-1-phosphate and inversion of the configuration from α to β form. The RO group represents the phenolic compound undergoing glucuronidation.
Whilst glucuronide conjugates are considered to be pharmacologically inactive, there is evidence to suggest that glucuronide conjugates can sometimes contribute to the activity of the parent compound. Indeed morphine-6-glucuronide has been found to be more potent as an analgesic agent than its parent compound, morphine, both in rats [Paul et al., 1989] and humans [Osborne et al., 1988]. Arakawa et al. compared the antitumour activity of 5-fluorouracil with its glucuronide conjugate in animals and found the conjugate to be more active [Arakawa et al., 1981].

There is accumulating evidence that UGTs exist in multiple forms in most species. In humans, 15 UGT isoforms have been identified [Tukey and Strassburg, 2000]. These can be divided into subfamilies, UGT1A and UGT2 encoded by the UGT1A locus and UGT2 genes respectively [Tukey and Strassburg, 2000].

The activity of UGTs is affected by several factors. Firstly, Kalow demonstrated that interethnic differences in glucuronidation exist [Kalow, 1989]. Secondly, age of the individual can affect the activity of UGTs [Dawling and Crome, 1989]. In neonates, glucuronidation is known to develop during the first few years of life [Boreus, 1989]. Thirdly, obesity has been associated with an enhanced capacity for biotransformations of drugs or endogenous compounds by glucuronidation [Abernathy et al., 1983]. Interestingly, several studies have shown that impaired hepatic function does not affect the glucuronidation of drugs [Ochs et al., 1986; Patwardhan et al., 1981].
1.4.2 Sulphation

Many compounds that undergo glucuronidation are also substrates for sulphation [Mulder, 1981]. Sulphotransferases (SULTs) catalyse the transfer of \( \text{SO}_3^- \) to both xenobiotic and endobiotic compounds. The cofactor, 3'-phosphoadenosine 5'-phosphosulphate (PAPS), required for these reactions (see Figure 1.7) is generated from inorganic sulphate (\( \text{SO}_4^{2-} \)) and adenosine 5'-triphosphate (ATP). PAPS is generated in the cytosol by two sequential reactions (see Figure 1.8). In the first step, adenosine 5'-phosphosulphate (APS) is generated from ATP by the action of ATP sulphurylase. The conversion of APS to PAPS is catalysed by APS kinase. In vivo, cysteine is the most common source of the sulphate that is required for PAPS synthesis. Consequently the low cellular levels of PAPS (~75 \( \mu \text{M} \)) compared to UDPGA (~350 \( \mu \text{M} \)) are due to the lack of availability of free cysteine. In the gut inorganic sulphate is also thought to originate from food [Mulder, 1981].

The human SULT family can be subdivided according to amino acid sequence and enzymatic function into subfamilies, SULT1 (phenol sulphotransferases) and SULT2 (steroid sulphotransferases) [Coughtrie et al., 1998]. Recently a new subfamily, SULT4A, has been identified in rat and human brain, although its function remains to be elucidated [Falany et al., 2000]. In humans, the SULT family is comprises at least 11 isoenzymes [Richard et al., 2001]. The SULT1 subfamily have between 47 and 96% amino acid sequence homology [Eisenhofer et al., 1999]. Some of the isoenzymes have alternative names, which are indicated in brackets. The SULT1 subfamily is comprised of SULT1A1 (P-PST), SULT1A2 (HAST 4), SULT1A3 (M-PST), SULT1B1 (ST1B2), SULT1C1 (ST1C2), and SULT1E1 (EST) [Eisenhofer et al., 1999]. These isoenzymes exhibit distinct, but overlapping substrate specificity for compounds of both external and internal origin. The SULT1A3 isoenzyme is
of particular interest, because it exhibits strong substrate specificity for endogenous biogenic amines, such as dopamine [Eisenhofer et al., 1999].
Figure 1.7. Chemical structure of PAPS

Figure 1.8. Synthesis of PAPS in vivo

\[
\begin{align*}
\text{SO}_4^{2-} + \text{ATP} & \xrightarrow{\text{ATP-sulphurylase}} \text{APS} + \text{PPi} \\
\text{APS} + \text{ATP} & \xrightarrow{\text{APS-kinase}} \text{PAPS} + \text{ADP}
\end{align*}
\]
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Generally the sulphation pathway is considered to be a detoxification pathway that has evolved to eliminate endogenous substrates. However certain sulphate conjugates are relatively unstable and degrade to form electrophilic carcinogens. In rodents, safrole is metabolised by cytochrome P450 enzymes to generate 1'-hydroxysafrole which is sulphated to generate the ultimate tumour-initiating compound, 1'-sulphoxysafrole [Boberg et al., 1983]. 1'-Hydroxysafrole is more active than safrole. There are two lines of evidence to support the fact that 1'-sulphoxysafrole is the ultimate electrophilic and carcinogenic compound. Firstly, the hepatotumourigenic effect of 1'-hydroxysafrole is inhibited if mice are treated with the sulphotransferase inhibitor pentachlorophenol. Secondly, 1'-hydroxysafrole has less affect on brachymorphic mice, which have a genetic defect in PAPS synthesis and consequently reduced capacity to sulphate xenobiotics.

1.5 Reduction of xenobiotic compounds

Holder et al. demonstrated that curcumin was reduced to form THC and HHC in male Sprague-Dawley rats [Holder et al., 1978]. Baker’s and brewer’s yeast are a convenient source of reductases that have been used to reduce ketone moieties [Roberts et al., 1985]. It is also possible to purify dehydrogenases from yeast and horse liver. However during the purification process, the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) dissociates from the enzyme. Consequently, reduction incubations using isolated enzymes must be fortified with a source of NADPH.
1.6 Aims

Despite the substantial amount of evidence for the chemopreventive activity of curcumin at both the tissue and cellular level as outlined in section 1.3.2 and 1.3.3 respectively, the role that metabolism plays in its efficacy is not understood. The overall aim of this project was to investigate the metabolism and bioavailability of curcumin. The results from this study should provide information that will be useful in the planning of clinical trials of this agent. The different parts of the work described in this thesis have the following inter-related objectives:

i) In order to quantify levels of curcumin and its putative metabolites in biological samples, a reversed phase high-performance liquid chromatography (HPLC) method with UV-visible detection was developed. In order to identify any putative metabolites, the HPLC method was adapted to ensure compatibility with electrospray ionisation mass spectrometry. To allow characterisation of putative metabolites identified by LC-MS, authentic reference compounds were synthesised or generated enzymatically. The putative metabolites curcumin sulphate, curcumin glucuronide and hexahydrocurcinolin (HHCOH) were generated and purified by preparative HPLC.

ii) Ravindranath et al. demonstrated that curcumin is biotransformed by rat gastrointestinal tissue (see section 1.3.4). Gut homogenate has also been shown to metabolise curcumin, although its metabolites have not been unambiguously identified [Asai et al., 2000]. An aim of this part of the work was to test the hypothesis that the gastrointestinal tract plays a role in curcumin conjugation and/or reduction. Subcellular fractions, such as microsomes and
cytosol are a suitable source of drug metabolising enzymes. The advantages of using subcellular fractions over whole cell systems include maintenance of enzymatic activity during storage at -80°C [Kronbach, 1995] and the ease of preparation from hepatic and intestinal tissue. The liver has a major role in phase I and II metabolism of xenobiotic compounds. In order to compare the extent of curcumin metabolism in the intestine with that in the liver, intestinal and hepatic metabolism of curcumin was quantified.

iii) The rat has been used for both in vitro and in vivo studies of curcumin metabolism, as described in section 1.3.4. Another objective of this work was to explore whether the rat is a suitable model to reflect curcumin metabolism in humans. To achieve this objective, reduction and conjugation of curcumin by hepatic and intestinal fractions were compared between tissues from rats and humans. In order to determine whether the metabolites of curcumin formed by subcellular gut fractions are also generated by intact gut tissue, curcumin was incubated with everted rat gut sacs.

iv) The liver has a major role in phase I and II metabolism of xenobiotic compounds. When isolated rat hepatocytes were incubated with curcumin, 90% of the parent compound was removed within 30 min, but the metabolites were not identified [Wahlstrom and Blenow, 1978]. Further two aims of the work described here were to establish the identity of hepatic metabolites and to determine whether there is a similar pattern of hepatic metabolism in rats and humans. Hepatocytes make up 80% of liver cellular content and are responsible for metabolism of both xenobiotic and endogenous compounds. The use of hepatocytes in biotransformation studies circumvents several of the shortcomings associated with using isolated enzymes or subcellular fractions. Firstly, cellular integrity is maintained and consequently xenobiotic metabolism by isolated hepatocyte suspensions is more analogous to
the in vivo situation. Secondly, the whole cells contain all hepatic enzymes, competing endogenous substrates and cofactors, therefore prior knowledge of the enzyme compartmentalisation and the necessary cofactors is not required.

v) In section 1.3.2 the ability of curcumin to prevent or reduce tumour incidence in vivo was discussed. In these studies, curcumin was administered to rodents as a dietary constituent (see section 1.3.2), whereas studies of this compound’s pharmacokinetics and metabolism have normally been performed using i.g. or i.p. routes of administration (see section 1.3.4). A further aim of the work was to determine whether the disposition of curcumin differs according to the mode of oral administration. Levels of parent compound and its metabolites in liver, colon mucosa and plasma were compared following administration of curcumin as a dietary constituent or by gavage.

vi) Curcumin is probably poorly absorbed from the alimentary tract (see section 1.3.4). Another aim of the work was to compare the absorption of curcumin following administration of the agent in different formulations or as a prodrug, in order to determine whether certain formulations/prodrugs might improve the oral bioavailability of the agent. The following three formulations/prodrug were administered to female F344 rats as a single i.g. dose and the plasma levels of curcumin and its putative metabolites were compared:

1) “Phytopharm P54” contains curcuminoids together with essential oils extracted from Curcuma Domestica and Xanthorrhiza plants. It was considered that the essential oils might improve the absorption of curcumin from the alimentary tract

2) Curcumin gamma linoleic acid (GLA) ester (see Figure 2.4). The GLA moiety requires hydrolysis before curcumin is released and it is therefore conceivable that this scenario may increase oral bioavailability
vii) Although metabolites of curcumin have been identified *in vitro* and *in vivo* (see section 1.3.4), the enzymes involved in the metabolic generation of these metabolites have not been identified. Another aim of the project was to characterise some of enzymes involved in curcumin biotransformation to HHC and curcumin sulphate. SULT1A1 and 1A3 are among six isoenzymes of the human phenol xenobiotic metabolising SULT1 subfamily that are expressed in the gastrointestinal tract [Eisenhofer *et al.*, 1999]. In order to determine whether these enzymes are involved in the metabolism of curcumin, the agent was incubated with recombinant SULT1A1 and 1A3. Immunoblot analysis was used to confirm that these isoenzymes are expressed in human gut and liver. In order to establish whether alcohol dehydrogenases may be involved in curcumin reduction, curcumin was incubated with alcohol dehydrogenase.

viii) A major objective of this project was to ascertain whether curcumin is absorbed and metabolised in humans. In order to test this hypothesis, the absorption and metabolism of curcumin was investigated in a pilot study of oral curcumin in humans. Patients suffering from colorectal cancer received curcuminoids “Phytopharm P54FP” formulation once daily. Faeces, urine and blood samples were analysed for curcumin and its putative metabolites. A major aim of this part of the work was to correlate the pharmacokinetic data with changes in surrogate biomarkers, which were determined in a related project undertaken by Dr. R. Sharma, University of Leicester. In an attempt to improve the bioavailability of curcumin in humans it was prepared as a microcrystalline formulation by contractors of the NCI. The microcrystallisation of compounds in order to increase surface area and decrease particle size is thought to improve oral bioavailability [Brenner *et al.*, 2001].
formulation underwent a pilot investigation in student volunteers conducted by Dr. D. Brenner, University of Michigan. The plasma of the volunteers was analysed, both in the US and at the University of Leicester. The aim of this study was to investigate the pharmacokinetics and safety of administration of a microcrystalline formulation of curcumin.
2 Materials and Methods
2.1.1 Chemicals and reagents

The following chemicals and reagents were purchased from the suppliers listed: curcumin, uridine 5'-diphosphogluconic acid (UDPGA), foetal calf serum (FCS), bovine serum albumin (BSA), tricarboxylic acid (TCA), uridine 5'-diphospho-N-acetyl glucosamine (UDPAG), magnesium chloride, 3'-phosphoadenosine 5'-phosphosulphate (PAPS), collagenase, 3'-phosphoadenosine 5'-phosphate (PAP), nicotinamide diphosphate (NADPH), N-napthylethylene diamine, 7-hydroxycoumarin, glycerol, penicillin/streptomycin, glycine, sodium hydroxide (NaOH), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES), dithiothreitol (DTT), uridine 5'-diphosphoglucuronosyl transferase, bacterial β-glucuronidase (type VII-A from *E. coli*), sulphatase (type VIII from Abalone entrails), sodium nitrite, ethylene diacetic acid (EDTA), 2-aminophenol, glycerol formal (60% 5-hydroxy-1,3-dioxane and 40% 4-hydroxymethyl-1,3-dioxalone), cremaphore, ethylene, equine alcohol dehydrogenase and triton X-100: Sigma-Aldrich Comp. Ltd. (Poole, Dorset, UK); curcumin used for feeding study: Apin Chemicals Ltd. (Abingdon, UK); mTHPC and curcumin gamma linoleic acid ester: Scotia Holdings plc (Fife, UK); HPLC-grade methanol, acetonitrile and ethyl acetate, diethyl ether, isoamyl alcohol and sodium acetate: Fisher Laboratory Suppliers Ltd. (Loughborough, UK); Hank's balanced saline solution (HBSS) concentrate without calcium, magnesium, sodium bicarbonate and phenol red, Dulbecco’s modified eagle medium (DMEM) with high glucose and glutamax, William’s E Medium, liver digestion medium, liver perfusion medium (DMEM with glutamax-1) and liver suspension medium (DMEM supplemented with 2% human serum albumin): Gibco Ltd. (Paisley, UK); rat plasma: Charles River (Margate, Kent, UK); pentobarbitone/ Sagatal: (Rhone Mérieux Ltd., Harlow, Essex, UK); sulphur-trioxide-N-triethylamine complex: Fluka Chemicals (Gillingham, Dorset, UK);
dimethyl sulfoxide and ammonium acetate: Merck Ltd. (Poole, Dorset, UK); halothane: Zeneca (Macclesfield, Cheshire, UK); enhanced chemiluminescence kit: Nycomed Amersham Pharmacia (Aylesbury, UK); Baxters Soltran kidney perfusion solution: Baxters Healthcare (Berkshire, UK); Bio-Rad protein assay dye reagent concentrate: Biorad Ltd (Bio-Rad Laboratories GmbH, Munich, Germany); C18 solid phase extraction columns: Varian (Walton-on-Thames, UK).

THC and HHC were synthesised as described [Uehara et al., 1987] and kindly provided by Dr. W. Wang, Phytopharm plc (Cambridge, UK). “Phytopharm P54” and “Phytopharm P54FP” used in the rat and human in vivo studies respectively were provided by Dr. I. Rubin, also of Phytopharm plc (Cambridge, UK). The recombinant sulphotransferase 1A1 and 1A3 enzymes [Dajani et al., 1998] and the monoclonal sulphotransferase 1A1/1A3 primary antibody [Richard et al., 2001] were a gift from Dr. M. Coughtrie, Ninewells Hospital & Medical School, University of Dundee (Dundee, Scotland).

Reversed phase C18 HPLC columns were purchased from the suppliers listed: Prodigy: Phenomenex (Macclesfield, Cheshire, UK); SymmetryShield: Waters (Watford, UK); Supercostl Elite and Shandon: Thermo-Hypersil (Runcorn, Cheshire, UK); ODS column: Beckman (High Wycombe, Buckinghamshire, UK).

2.1.2 Cells and animals

Nonmalignant human colon epithelial cells (HCEC) were obtained from Dr. A. Pfeifer, Nestle Research Institute (Lausanne, Switzerland) and the malignant colon tumour cells, SW 480, HT-29 and HCA-7 were obtained from C. Paraskeva, Bristol University (Bristol, UK) and Ms
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S. Kirkland, Imperial College (London, UK) respectively. These cells were passaged in Dulbecco's modified eagle medium (DMEM) with high glucose and glutamax. Human hepatocytes were prepared from healthy human liver obtained by Dr. S. Orr, UK Human Tissue Bank (Leicester, UK). Male (180-200 g) or female (160-180 g) F344 rats were purchased from Charles River UK Ltd. (Margate, Kent, UK) or Harlan UK Ltd. (Bicester, Oxon, UK). Rats were kept in a purpose-built animal house in negative pressure isolators (19-23°C) under a 12-hr light/dark cycle. The rats received RM1 rodent maintenance diet (SDS, Kent, UK) and water ad libitum. Experiments using animals were conducted as stipulated by Project Licence 80-1250 granted to the Medical Research Council Toxicology Unit by the UK Home Office. The experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation.

2.2 Source of curcumin

Commercially available curcumin also contains the impurities desmethoxycurcumin and bisdesmethoxycurcumin (see Figure 2.1). The ratio of curcumin to the impurities depends on the source used. Curcumin purchased from Sigma-Aldrich Comp. Ltd. was found to contain 10% desmethoxycurcumin and 2% bisdesmethoxycurcumin by HPLC. The curcuminoids were identified by mass spectrometry in the selected ion mode. Curcumin was also obtained from Apin Chemicals Ltd. and found to contain 9% desmethoxycurcumin, but no bisdesmethoxycurcumin. "Phytopharm P54" and "Phytopharm P54FP" were obtained from Phytopham plc and found to contain 10% desmethoxycurcumin, but no bisdesmethoxycurcumin. It is not clear whether desmethoxycurcumin and bisdesmethoxycurcumin contribute to the chemopreventive efficacy of curcumin.
Figure 2.1. Chemical structures of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin
2.3 Synthesis of curcumin metabolites

2.3.1 Synthesis of curcumin sulphate and curcumin glucuronide

For the synthesis of curcumin sulphate, curcumin (1.36 mmol), dissolved in anhydrous 1,4-dioxane, was reacted with sulphur trioxide N-triethylamine complex (6.8 mmol) and maintained at 37°C for 2 hr. The precipitate was washed with ethyl acetate in order to remove unreacted curcumin. For the biosynthesis of curcumin glucuronide according to a published method [Manach et al., 1998], curcumin (1 mM), UDPGA (4 mM), UDPAG (2 mM), HEPES buffer (25 mM, pH 7.4), magnesium chloride (10 mM) and uridine 5'-diphosphoglucuronosyl transferase (150 units/l) were incubated (3 ml, 37°C, 180 min). The incubation medium was extracted twice with ethyl acetate, and the combined organic extracts were evaporated under nitrogen. The residues of either reaction were reconstituted in acetonitrile:water (1:1, v/v). Curcumin conjugates were separated from parent compound by preparative HPLC using a C18 Hypersil column (BDS, 250 × 21.2 mm, 5 μm, detection at 420 nm). Eluent corresponding to the peaks which were tentatively assigned to curcumin sulphate or curcumin glucuronide was collected, and the mobile phase removed from the collected fractions by freeze-drying. The isolated materials were re-analysed by HPLC with detection at 420 nm. The purity of curcumin sulphate and curcumin glucuronide was determined to be 98 and 99%, respectively. The structural identities of the products as curcumin sulphate and curcumin glucuronide was confirmed by mass spectrometry.
2.3.2 Synthesis of hexahydrocurcuminol.

HHCOH was generated from HHC using the reducing agent sodium borohydride. An equimolar amount of sodium borohydride was added to HHC (3 mM) dissolved in methanol and maintained at room temperature. HPLC analysis with detection at 280 nm showed that after 2 hr at ambient temperature all of the HHC had disappeared. Methanol was removed by evaporation under nitrogen and the residue was reconstituted in water (2 ml) and adjusted to pH 4.5. The product was extracted with ethyl acetate and the solvent evaporated under nitrogen. The structural identity of the product as HHCOH was confirmed by mass spectrometry.

2.4 Development and validation of the HPLC method

Several groups have published methods for the detection of curcuminoids in biological samples [Taylor and McDowell, 1982; Singh et al., 1981; Pan et al., 1999]. A reversed phase (RP) HPLC method with UV/visible detection was used to determine the quantity of curcumin and its putative metabolites that was similar but not identical to that described previously [Pan et al., 1999]. A Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (Varian 310 model) and autosampler (Varian 410 model) were used. The maximal absorbance wavelength of curcumin, curcumin sulphate and curcumin glucuronide was established by UV spectroscopy to be 420 nm, whereas 280 nm was found to be the optimal absorbance wavelength for the detection of THC, HHC and HHCOH. The separation of the curcuminoids, curcumin, desmethoxycurcumin and bisdesmethoxycurcumin was compared using four different C18 reversed phase (RP) columns which were packed with either
octadecyl silane (ODS) or base deactivated silica (BDS): (1) Supercostl Elite (BDS, 250 x 4.6 mm, 5 μm), (2) Prodigy (ODS, 150 x 4.6 mm, 5 μm), (3) Shandon (BDS, 250 x 4.6 mm, 5 μm) and (4) Waters SymmetryShield (ODS, 150 mm x 4.6 mm, 5 μm). The Waters SymmetryShield column was found to provide optimal resolution. Interestingly, the elution order of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin was reversed by the Waters column compared to the other three C18 columns.

In order to quantify levels of curcumin and its metabolites in biological samples, it was necessary to use an internal standard. By using an internal standard it is possible to avoid experimental error due to incorrect injection onto the HPLC, variation in the sensitivity of the analytical instrument and dilution of the sample. Any alterations that occur that affect the sample will affect not only the compound of interest, but also the internal standard. mTHPC was an ideal candidate as an internal standard for two reasons. Firstly, it has been demonstrated to absorb at both 420 and 280 nm and secondly under the HPLC conditions used it does not co-elute with curcuminoids or endogenous plasma components.

To improve the resolution and separation of the curcuminoids, mTHPC, and putative metabolites, a systematic trial of mobile phase conditions was necessary. Solutions of curcumin dissolved in acetonitrile: water (1:1, v/v) were injected on to the HPLC column. Firstly, methanol and acetonitrile were compared as the constituents of the mobile phase. Secondly, it was shown that including a buffer in the mobile phase significantly improved the resolution of the curcuminoids. Thirdly, the optimal concentration and pH of the ammonium acetate buffer was determined to be 0.01 M and pH 4.6 respectively.
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Silica columns, such as the Waters column, can become contaminated with metal ions. Metal ions can affect the elution of compounds from the column and may cause broader peaks and peak tailing. Flavonoids such as quercetin chelate metal ions resulting in poor resolution and peak symmetry [Jones et al., 1998]. The addition of a metal ion chelator, e.g. EDTA, to the mobile phase could potentially improve peak separation and shape [Jones et al., 1998]. EDTA was added to the mobile phase and found not to affect peak resolution or peak shape.

A linear gradient of 5-45% acetonitrile in 0.01 M ammonium acetate (pH 4.6) for 30 min was used, followed by an increase over 20 min to 95% organic component. The flow rate used was 1.0 ml/min and the injection volume was 100 µl. These conditions will be referred to in the text as the “gradient HPLC method”.

In order to protect the analytical column, a Waters C18 guard column was used. The packing of this column was identical to that of the analytical column. The guard column was typically replaced at intervals of 10 weeks or as was necessary.

Algae often grows in the mobile phase of HPLC systems. Three steps were taken to reduce contamination of the mobile phase. Firstly, HPLC grade reagents and water were used. Secondly, a 1 M stock solution of ammonium acetate was prepared and diluted immediately prior to use. Thirdly, all mobile phase constituents were routinely filtered.

Blood was taken from patients at Leicester Royal Infirmary (L.R.I.), transferred to tubes containing lithium heparin, and pooled. Plasma was immediately separated from blood cells by centrifugation (2,800 × g, 4°C, 15 min) and stored at -80°C. Plasma (1.0 ml) was spiked
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with curcumin or its putative metabolites and serially diluted to the required concentrations with control plasma. Curcumin was dissolved in acetonitrile: water or DMSO and the total solvent content in the plasma was calculated to be <0.1%. The solutions were allowed to reach equilibrium at ambient temperature in the dark.

Aliquots of plasma (100 µl) were spiked with mTHPC, mixed with 200 µl of methanol: DMSO (4:1, v/v) and vortexed for 30 sec. The resulting mixture was cooled on ice for 30 min, to precipitate proteins. The tubes were centrifuged (17,060 x g, 4°C, 15 min) and the supernatant removed. Prior to injection, samples were diluted (1:1, v/v) with water. Peak areas were determined by integration using Star Chromatography software version 5.13. The limit of detection is the lowest concentration of analyte that can be reliably differentiated from background levels [Shah et al., 1992]. Generally the ratio of analyte: baseline noise should not be less than 3:1. The limit of detection of curcumin in plasma was determined to be 0.01 µg/ml. The recovery of curcumin from plasma at 0.1 µg/ml and 10 µg/ml was determined to be 70 ± 5% and 72 ± 5% respectively (mean ± SD, n = 3).

In order to improve the detection limit of curcumin in plasma, the extraction procedure was modified. Plasma (1 ml) was spiked with mTHPC, extracted twice with ethyl acetate (2 ml) and mixtures were centrifuged (2800 x g, 4°C, 15 min). If protected from light, curcumin is stable in ethyl acetate for 10 hrs [Tonnesen and Karlsen, 1985]. The organic layers were removed, combined and immediately evaporated to dryness at room temperature under nitrogen. Nitrogen was used for solvent removal instead of rotary evaporation, in order to avoid potential degradation of curcuminoids at 40°C [Wang et al., 1997]. Samples were reconstituted in acetonitrile: water (1:1, v/v) and analysed by HPLC. To determine the extraction efficiency of curcumin and its metabolites, plasma was spiked with curcuminoids
to give final concentrations of 0.1 and 40 μg/ml. The peak areas resulting from the plasma extracts were compared with those resulting from standard solutions. The extraction efficiencies for curcumin, HHC and curcumin sulphate were 95 ± 4%, 70 ± 5%, and 7 ± 5 respectively (mean ± SD, n = 6).

In order to improve the extraction efficiency of curcumin sulphate, the plasma was acidified to a range of pH values by addition of cold 1 M acetate buffer (2 ml). When the plasma was acidified to pH 4.6 prior to extraction with ethyl acetate: propan-2-ol (9:1, v/v), the extraction efficiency of curcumin sulphate was found to be 49 ± 9%. Cleavage of the sulphate moiety was observed under more acidic conditions. Extraction efficiencies of curcumin, curcumin glucuronide, HHC and curcumin sulphate from plasma were determined by HPLC at 0.1 μg/ml and 40 μg/ml (see Table 2.1). The limits of detection and quantification of curcumin in plasma were determined to be 0.1 ng/ml and 0.01 μg/ml respectively. Curcumin calibration curves spanned the concentration range of 0.01 μg/ml and 40 μg/ml. The intra-day and inter-day coefficients of variation (Standard deviation ÷ Mean × 100) for determination of curcumin in plasma were calculated at 0.1 and 40 μg/ml (n = 6) (see Table 2.2).

An emulsion was frequently formed between the organic layer and the plasma layers during the ethyl acetate extraction process. The magnitude of the emulsion layer could be decreased by using chilled and water-saturated ethyl acetate: propan-2-ol for extraction. In addition, tubes were thoroughly vortexed between centrifugation steps to disaggregate the emulsion formed.
Table 2.1. The extraction efficiencies (mean % ± SD) of curcumin and its metabolites from plasma at 0.1 and 40 μg/ml (n = 6). Detection of curcumin glucuronide, curcumin sulphate and curcumin was achieved at 420 nm and gradient HPLC conditions were used (see section 2.4). HHC was detected at 280 nm. Chromatographic peaks were identified by co-chromatography and LC-MS.

<table>
<thead>
<tr>
<th>Curcuminoid</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 μg/ml</td>
</tr>
<tr>
<td>Curcumin glucuronide</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>Curcumin sulphate</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>HHC</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>Curcumin</td>
<td>92 ± 7</td>
</tr>
</tbody>
</table>

Table 2.2. Intra and inter day coefficients of variation (n = 6).

<table>
<thead>
<tr>
<th>Type of variation</th>
<th>Coefficients of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 μg/ml</td>
</tr>
<tr>
<td>Intra-day</td>
<td>17</td>
</tr>
<tr>
<td>Inter-day</td>
<td>19</td>
</tr>
</tbody>
</table>
2.4.1 Stability of curcumin and mTHPC

It has been suggested by Wang et al. that curcumin is rapidly degraded at room temperature [Wang et al., 1997]. Determination of the stability of curcumin and mTHPC in acetonitrile: water (1:1, v/v) was carried out by maintaining aliquots of standard solutions (0.1 and 40 µg/ml) at room temperature. These aliquots were analysed by HPLC after 1, 2, 3, 4 and 8 hr. No significant change in the concentration of the solutions was observed over a period of 8 hr. Consequently, 8 hr was the maximum amount of time that samples were maintained in the autosampler prior to analysis.

Stock solutions of curcumin (1 mg/ml) and mTHPC (1 mg/ml) dissolved in DMSO were prepared each week and stored at +4°C. Curcumin and mTHPC were both found to be stable when maintained under these conditions.

Human plasma was spiked with curcumin (0.1 and 40 µg/ml) and maintained at -80°C for three months. Under these storage conditions, curcumin was found to be stable. Indeed solutions of curcumin in methanol have been shown to be unchanged following storage at -70°C for one year [Tonnesen and Karlsen, 1985]. Three months was the maximum amount of time that plasma samples were stored prior to analysis. The influence of freeze/thaw cycles on curcumin stability in plasma was not investigated as patient samples were routinely stored in small aliquots and only thawed once.
2.5 Development of a mass spectrometry method

Mass spectrometric analysis of curcumin and its metabolites was achieved using a Quattro Bio-Q tandem quadrupole mass spectrometer upgraded to Quattro MK II specifications (Micromass, Altrincham, Cheshire, UK) with a pneumatically assisted electrospray interface. Samples were analysed in the negative ion mode. The temperature was maintained at 120°C; the operating voltage of the electrospray capillary was 3.88 kV and the cone voltage 32 V. Tandem mass spectrometric experiments were conducted using argon as the collision gas and collision energy of 25 eV. Samples were dissolved in acetonitrile: water (1:1, v/v) and introduced into the mass spectrometer via flow injection using a Varian 9012 solvent delivery system (Varian, Walton-on-Thames, UK) and a Rheodyne 7125 injector (Cotatai, Ca, US). HPLC conditions were as described in section 2.4, except in certain experiments the linear gradient programme was: acetonitrile (5-45%) in 0.01 ammonium acetate (pH 4.6) for 60 min as opposed to 30 min, followed by an increase for 20 min to 95% acetonitrile. The HPLC flow rate used was 1 ml/min. To reduce the flow rate into the mass spectrometer to 115 μl/min, the flow was split using a post column T piece (1:9) with the excess flowing to waste. In some experiments the solution was introduced into the mass spectrometer by continuous infusion using a Harvard Apparatus model 22 syringe pump (Harvard apparatus, South Natick, MA, US). The flow rate used was 10.0 μl/min.
2.6 Metabolism of curcumin by intestinal and hepatic subcellular fractions

2.6.1 Preparation of intestinal and hepatic microsomes and cytosol

Male F344 rats were subjected to terminal anaesthesia (halothane/nitrous oxide) and blood was removed by cardiac puncture. Animal handling, terminal anaesthesia and dissection of animals were performed under project licence 80/1250. Liver and intestine were removed and snap frozen in liquid nitrogen. Tissue was resected from 6 caucasian patients (liver from 1 male who was 4 years of age, 2 females who were 30 and 51 years old, and intestine from 3 females who were 29, 54 and 56 years of age). Patients had not received medication known to interfere with liver metabolic activity. Human and rat intestinal or hepatic tissue was defrosted, weighed and minced using scissors. Microsomes and cytosol were prepared from tissue by differential centrifugation as described previously [Adams et al., 1985]. Essentially, tissue was suspended in ice-cold 0.01 M KCl-phosphate-EDTA buffer (11.5 g KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM pefabloc), homogenised for 10 sec (Ultraturax T25-57) and centrifuged (14,500 × g, 4°C, 20 min). The supernatant was removed and ultracentrifuged (105,000 × g, 4°C, 60 min). The resulting supernatant (cytosol) was immediately snap frozen in liquid nitrogen and the pellet resuspended in buffer and ultracentrifuged (105,000 × g, 4°C, 40 min). Pelleted material was resuspended in sucrose buffer (approximately half original volume) (100 mM TrisHCl, 250 mM sucrose and 10% glycerol) and snap frozen. Human intestinal tissue originated from the jejunal area of the small intestine and rat intestinal tissue from the jejunum and the colon.
2.6.2 Determination of sample protein concentration

The protein content of samples was determined using the Biorad assay which is a modified version of the method originally developed by Bradford [1976]. This method is based on the change in colour of Commassie Brilliant Blue G-250 (Biorad reagent) in response to various concentrations of protein. When protein binds to Biorad reagent, the absorbance maximum of the acidic solution shifts from 465 to 595 nm. Therefore, by application of the Beer-Lambert Law, the protein content of a sample may be determined. Samples were diluted with water (1:500, v/v) and mixed thoroughly. A stock solution (1 mg/ml) of BSA was prepared and diluted to a range of concentrations between 0.5 and 20 μg/ml with HPLC grade water. Biorad reagent (200 μl) was added to the sample or standard solution (800 μl) and the spectrophotometric absorbance at 595 nm determined. The protein concentration of samples was determined by comparison to the calibration curve.

2.6.3 Purification of PAPS

Commercially available PAPS is often accompanied by a contaminant, PAP, which is an inhibitor of sulphotransferases [Marcus et al., 1980]. Therefore the purity of the purchased PAPS was determined by HPLC and found to be 80%. In order to remove PAP, the commercially available PAPS was purified by HPLC, essentially as described previously [Boocock et al., 2000] with some modifications. A Varian Prostar (310 model) solvent delivery system was used, coupled to an ODS reversed phase C18 column (250 × 4.6 mm; 5 μm) and a UV-visible detector. Aliquots (100 μl) of the PAPS solution (4 mM) were injected on to the column, and the eluent flow rate was 1.3 ml/min. The eluent containing PAPS was
collected on dry ice and concentrated by rotary evaporation for approximately 4 min at ambient temperature. The collected PAPS was determined to be >99% pure by HPLC.

2.6.4 Incubation of curcumin with subcellular fractions

Curcumin conjugation was investigated in incubates of hepatic or intestinal cytosol or microsomes (1 mg cytosolic or microsomal protein per ml) with curcumin (100 μM) and cofactors in phosphate buffer (0.01 M, pH 7.4 for glucuronidation and pH 8.4 for sulphation) at 37°C for 1 hr. The final volume of incubates was 0.5 ml. The maximum concentration of DMSO added to each incubate was < 0.1%. The concentration of substrate was selected for two reasons. Firstly, 100 μM was a sufficiently high concentration so that minor metabolites could be detected by HPLC and LC-MS. Secondly, the concentration was in the order of the concentrations used in cellular work carried out by others working at the MRC Toxicology Unit. Incubations to study glucuronidation included microsomes with UDPGA (3 mM), magnesium chloride (4 mM) and triton X-100 (0.01 %). Incubations to study sulphation included cytosol with PAPS (0.4 mM) and mercaptoethanol (5 mM). The stability of curcumin in 0.01 M phosphate buffer (pH 7.4, and 8.4) maintained at 37°C for 2 hr was determined. Under these incubation conditions, there was a negligible loss in parent compound, which is contradictory to the observations of Wang et al. (see section 1.3.1). The authors found that when curcumin was maintained at 37°C in 0.1 M phosphate buffer for 30 min, 90% of the agent degraded [Wang et al., 1997]. The extinction coefficients of curcumin sulphate and curcumin glucuronide were approximately equivalent to that of curcumin, as established by a standard curve. Consequently the amounts of these metabolites were estimated with the help of a calibration curve established using curcumin (see Figure 2.2). In orientation experiments, curcumin glucuronide and sulphate (see Figure 2.3) generation was
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found to be linear for up to 10 min after which the rate declined. Therefore the values calculated are not rates. To study curcumin bioreduction, substrate (100 μM) was incubated with cytosol and NADPH (10 mM) in phosphate buffer (0.01M, pH 7.4) in a final volume of 0.5 ml at 37°C. Incubation time was 90 min (see Figure 2.3), during which generation of HHC was linear. The amount of HHC generated was assessed using a calibration curve with authentic HHC. In control experiments, microsomal or cytosolic fractions containing inactivated enzymes following boiling for 10 min were incubated with substrate and reaction components. As further controls, incubations were carried out omitting either substrate or subcellular fraction. Reactions were terminated by storing incubate samples on dry ice. Curcumin and its metabolites were extracted from the incubation media with ethyl acetate: propan-2-ol (see section 2.4) and analysed using the “gradient HPLC conditions” and mass spectrometric method described in sections 2.4 and 2.5 respectively. The extraction efficiencies of curcuminoids from phosphate buffer were found to be similar to that for plasma (see section 2.4).
Figure 2.2. Calibration curve used to calculate the amount of curcumin in phosphate buffer. Phosphate buffer (pH 7.4) was spiked with curcumin (at various concentrations) and mTHPC, extracted and analysed by HPLC as described in section 2.4. The regression coefficient was determined to be 0.999.
Figure 2.3. Time course of formation of curcumin glucuronide (▲), curcumin sulphate (■) and HHC (▼). Curcumin sulphate and HHC were generated by incubation of curcumin (100 µM) with human intestinal cytosol (1 mg protein per ml) respectively. Curcumin glucuronide was generated by incubation of curcumin (100 µM) with intestinal microsomes (1 mg microsomal protein per ml). Reactions were fortified with appropriate cofactors (see section 2.6.4). Curcumin and its metabolites were extracted with ethyl acetate: propan-2-ol (9:1, v/v). Analysis of curcumin and its metabolites was achieved using the gradient HPLC conditions described in section 2.4. Detection of curcumin sulphate and curcumin glucuronide was achieved at 420 nm, whereas 280 nm was the detection wavelength for HHC. The values calculated were the mean ± SD following preparation of microsomes and cytosol from separate individuals (n = 3).
The data was analysed using analysis of variance (ANOVA) general linear model and Excel and Minitab (Windows 2000) software packages. Post hoc Fisher’s Least significant Difference Test was performed.

2.7 Metabolism of curcumin by intact rat gut

The jejunal section of the intestine of terminally anaesthetised male F344 rats (180 g) was excised. Gut content was removed by flushing thoroughly with 0.9% (w/v) sodium chloride solution. A glass rod (150 mm × 3 mm) was used to push the ileal section of the small intestine through the gut lumen until it emerged from the duodenum and the intestine was everted [Wilson and Wiseman, 1954]. Everted gut sacs (approximately 8 cm in length) were tied at both ends and suspended in a modified Ussing chamber in Krebs-Ringer phosphate buffer (10 ml) as described previously [Ravindranath and Chandrasekhara, 1981]. The purpose of everting the gut was to allow maximal exposure of intestinal mucosa to curcumin. Krebs-Ringer phosphate buffer was prepared and saturated with carbogen (oxygen/CO₂ 5%) gas. The buffer contained glucose (10 mM) and curcumin (dissolved in DMSO) to give a final concentration of 100 μM. The total amount of DMSO added to the incubations was < 0.2%. Buffer was added to the gut sacs with a small needle in order to ensure that they were fully distended. Gut sacs were maintained in a continual stream of carbogen for 30, 60 or 180 min at 37°C. Mucosal fluid was sampled (2 ml) after 30 and 60 min and replenished with curcumin containing medium. Following termination of the experiment, gut sacs were homogenised in acetate buffer (pH 4.6). The homogenised gut sacs, the serosal media and the mucosal media were extracted with ethyl acetate: propan-2-ol and analysed using the “gradient HPLC conditions” described in section 2.4 and LC-MS as detailed in section 2.5.
2.8 Metabolism of curcumin by isolated rat and human hepatocytes

2.8.1 Isolation of human and rat hepatocytes

Hepatocytes were isolated from rats or humans using the two-step perfusion method [Seglen, 1976]. The first step involved perfusion of the liver with buffer devoid of Ca\(^{2+}\) in order to irreversibly cleave desmosomal cell to cell interactions. In the second step, collagenase was added to the perfusion buffer to digest the extracellular matrix. This method is a refined version of the original one-step perfusion method [Berry and Friend, 1969]. Healthy liver tissue was resected from 4 Caucasian patients with secondary hepatic tumors (2 females 38 and 61 years old, 2 males 51 and 53 years old). Patients had not received medication known to interfere with liver metabolic activity. Following removal of the liver from the body, cannulae were immediately inserted into 4-5 large blood vessels of the lobe, which was immediately perfused in theatre with kidney perfusion medium (500 ml) and transported in this fluid on ice. The liver was transferred to a custom-built stainless steel tank and perfused for 20-30 min with liver perfusion medium and maintained at 37°C. The liver was then perfused with liver digestion medium for approx. 45 min. The digested liver lobe was transferred to a tray containing liver suspension medium (DMEM supplemented with human serum albumin 2%). The medium also contained penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The tissue was gently disrupted in order to release cells. Undigested tissue was removed by passing the cell suspension through a series of sieves (successive mesh size 1 mm, 0.5 mm and 100 μm). For the isolation of rat liver cells, male F344 rats (180-220 g) were anaesthetised with an i.p. injection of sodium pentobarbitone (60 mg/kg). The liver was perfused (5 min, rate: 50 ml/min) via the inferior portal vein with HBSS (containing EGTA 1 mM), which had been pre-saturated with carbogen. The liver was digested using collagenase
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(100 mg/l) and calcium chloride (332 mg/l) in HBSS. Tissue was gently disrupted and washed through a sieve (100 μm mesh size) with liver suspension medium or William’s E medium. The medium also contained penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Human or rat cells thus obtained were washed three times and centrifuged (50 × g, 4°C, 3 min). Cells were counted using a haemocytometer immediately following isolation. Hepatocyte viability determined by the trypan blue exclusion assay was routinely 80% or above. Hepatocytes in suspension were maintained on ice for a maximum of 30 min prior to use.

Ideally, it would be convenient to store hepatocytes at -80°C in preparation for use. Hepatocytes were frozen in medium with addition of glycerol (10%) as a cryoprotectant. This concentration of glycerol was found not to be toxic to cells [Orr, personal communication]. Following thawing of the cells, there was insufficient viability (<20%) to warrant usage for metabolism studies.

2.8.2 Incubations with hepatocytes

Freshly isolated hepatocytes (2 x 10^6 cells per ml) were suspended in liver suspension medium (2 ml) and incubated at 37°C in a slowly shaking incubator. Curcumin dissolved in DMSO was added to give a final concentration of 100 μM. This substrate concentration was chosen in order to maximise the amount of metabolites that could be generated. The maximum incubate concentration of DMSO was 0.1% (v/v), which did not interfere with cell viability. Control incubates included curcumin with media alone, heat-inactivated hepatocytes, or intact hepatocytes with the vehicle only. Incubations were terminated after 5, 30, 60 and 120 min by placing vials on dry ice. Viability of incubates containing curcumin
and hepatocytes decreased to between 40 and 60% of its initial values (trypan blue exclusion test) during the longest incubation period which was 120 min. Samples were stored at -80°C in preparation for analysis by HPLC. Samples were extracted with ethyl acetate: propan-2-ol and analysed using the “gradient HPLC conditions” and mass spectrometry method described in sections 2.4 and 2.5 respectively. Extraction efficiencies of curcuminoids from hepatocyte suspensions were similar to those determined for plasma.

2.8.3 Assay for glucuronosyl transferase activity

Two methods were used to assess the ability of the hepatocytes to conjugate a model substrate. The first assay, developed by Bratton and Marshall [1939] relies on the diazotisation of the free amino group in 2-aminophenol by its reaction with sodium nitrite. The resulting diazonium salt was reacted with an aromatic amine (N-napthylethylene diamine) to produce an azo compound that was analysed spectrophotometrically at 540 nm. The method specifically detects 2-aminophenol glucuronide because the substrate, 2-aminophenol, is destroyed under the reaction conditions (pH 2.7) and consequently does not take part in the reaction. The method used was essentially as described previously [Gibson and Skett, 1999], except that triton X-100 was omitted from the reaction because of its potential hepatocytic toxicity. The second method was based on the spectrophotometric analysis of the fluorescent compound 7-hydroxycoumarin (umbelliferone) [Chipman et al., 1997]. Freshly isolated rat hepatocytes (2 × 10^6 cells/ml) or boiled hepatocytes (2 × 10^6 cells/ml) were incubated with 7-hydroxycoumarin (100 µM in DMSO) for 60 min at 37°C. Incubations were performed in duplicate. The reaction was terminated by adding 1.5 ml of incubate to 2 ml of H_2O and 1 ml of 0.2 M acetate buffer pH 4.5. The acidified samples were extracted with 4 ml diethyl ether containing 1.5% isoamyl alcohol and 2 ml of the organic
phase was back-extracted into 4 ml of 0.4 M glycine/NaOH buffer, pH 10.4. The fluorescence ($\lambda_{\text{excitation}}$ 370 nm and $\lambda_{\text{emission}}$ 450 nm) of the fresh hepatocyte extracts was calculated and expressed as a percentage of fluorescence of the boiled hepatocytes. As a positive control, microsomal protein (1 mg microsomal protein per ml) was incubated with UDPGA (3 mM), magnesium chloride (4 mM) and triton X-100 (0.01 %) and incubated with 7-hydroxycoumarin (100 $\mu$M in DMSO) at 37°C for 60 min. The resulting samples were extracted in the same way as isolated hepatocytes.
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2.9 Metabolism of curcumin by colonic cell line cytosol and isolated enzymes

2.9.1 Cell culture

The malignant colon cell lines (HCA-7, SW 480, HT-29) and the nonmalignant colon cell line (HCEC) were routinely passaged in medium (DMEM with high glucose and glutamax) containing FCS (10%). Cells were grown until reaching approximately 70% confluence.

2.9.2 Preparation of cellular cytosol

Cells were removed from the plates by addition of trypsin (1×) and incubation at 37°C. The trypsin was diluted with cell media (1:1) and centrifuged (300 × g, 20°C, 5 min). The pelleted material was resuspended in cell media and the total cell number was routinely determined to be in the order of 1×10⁷-1×10⁸. Following a second centrifugation step (300 × g, 20°C, 5 min) the pellet was resuspended in 0.5 ml swelling buffer (10 mM KCl, 30 mM tris base, 5 mM magnesium acetate, 5 mM EGTA and 10 mM benzamidine) and incubated on ice for 10 min. A hand-held homogeniser (10 strokes) was used to lyse the cells and the resulting homogenate was laid over 200 μl swelling buffer (containing 25% glycerol) and centrifuged (100,000 × g, 4°C, 60 min). The supernatant was removed and stored at -80°C.
2.9.3 Incubation of curcumin with isolated enzymes/cytosol

Curcumin (100 μM) was incubated with recombinant human SULT 1A1 and 1A3 (10 μg/ml). These isoenzymes have been shown to have substrate specificity for simple phenols and are expressed in human liver and gut [Eisenhofer et al., 1999]. Indeed it has been demonstrated that SULT1A3 metabolises vanillin, a degradation product of curcumin (see section 1.3.1) [Eisenhofer et al., 1999]. The recombinant SULT1A1 and SULT1A3 enzymes were prepared as described previously [Dajani et al., 1998]. Curcumin was also incubated with colonic cell line cytosol (1 mg cytosolic protein per ml), baker’s yeast or with equine alcohol dehydrogenase (10 U/ml). Incubations were carried out in phosphate buffer (0.01 M, pH 8.4 for sulphation, pH 7.4 for reduction). The final volume of each incubate was 0.5 ml. Incubations contained PAPS (0.4 mM, sulphation) or NADPH (10 mM, reduction) and were maintained at 37°C for 60 min. Metabolites were extracted with ethyl acetate: propan-2-ol and analysed using the “gradient HPLC conditions” and mass spectrometry method described in sections 2.4 and 2.5 respectively. The amount of curcumin sulphate generated by the human recombinant enzymes and colonic cell line cytosol was determined using a curcumin sulphate standard curve and mTHPC as an internal standard.

2.9.4 Immunoblot analysis

Cytosolic proteins (30 μg) were resolved by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (130 V) and transferred to a nitrocellulose membrane (100 V, 120 min) as described previously [Laemmli, 1970; Towbin et al., 1979]. A 12% running gel, 5% stacking gel, loading buffer, tris-glycine electrophoresis buffer and transfer buffer were prepared as described previously [Maniatis et al., 1989]. The membrane was washed with two aliquots of
TBST (0.02%), blocked in 5% milk for 120 min and incubated with the primary SULT1A3 antibody (1:1000, v/v) in TBST containing 5% milk for 120 min. The primary antibodies against recombinant human SULT1A3 [Dajani et al., 1998] were raised in sheep by three separate immunisations and were purified by column chromatography [Richard et al., 2001]. Following five washes in TBST (5 min), the membrane was incubated with the anti-sheep secondary antibody (1:4000, v/v) in TBST containing 5% milk for 120 min. Immunochemical detection of SULT1A1 and SULT1A3 proteins of immunoblots of human tissue cytosol was achieved using the enhanced chemiluminescence method (horseradish peroxidase) as described by the manufacturer (Nycomed Amersham Pharmacia plc).

2.10 Metabolism of curcumin in vivo in rats

2.10.1 Intravenous and intragastric administration of curcumin

Female F344 rats received curcumin via the i.v. route (40 mg/kg, vehicle: glycerol formal; dosage volume: 1.0 ml/kg) or by i.g. intubation (500 mg/kg, vehicle: DMSO; dosage volume: 2.0 ml/kg). The i.v. dose was chosen because the curcumin was made up in the largest amount of solvent that could be injected safely. The i.g. dose was selected because preliminary feeding studies revealed that it was approximately equivalent to administration of 1% curcumin in the diet, a dosage that has frequently been used in chemopreventive studies [Inano et al., 1999; Inano et al., 2000]. Animals were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture 5 and 30 min and 1 and 3 hr after i.v. dosing and 30 min, 1, 3, 6, 12 and 24 hr following i.g. dosing. Blood was also obtained from animals which had received vehicle only. Blood was transferred into heparinised centrifuge tubes, and plasma obtained by centrifugation (2800 x g, 4°C, 15 min). Aliquots of plasma were extracted with twice the volume of ethyl acetate, or mixed with four
times the volume of a mixture of DMSO:methanol (1:4, v/v) and analysed using the “gradient HPLC conditions” and mass spectrometry method described in sections 2.4 and 2.5 respectively. Curcuminoids were quantified by spiking rat plasma with curcumin and preparing a curcumin calibration curve.
2.10.2 Comparison of oral and intragastric administration of curcumin

Administration of dietary curcumin was compared with that of an i.g. bolus. For oral administration, curcumin (2%) was mixed into the food. For administration by gavage, curcumin was suspended and partially dissolved in a mixture of glycerol formal: cremophore: water (5:2:2, v/v/v), which in preliminary experiments was found to yield a suitable formulation in terms of acceptable viscosity and stability of suspension. This formulation was administered at 500 mg curcumin/kg (dosage volume: 2.0 ml/kg) by i.g. gavage once or daily for 7 consecutive days. This formulation was used instead of the DMSO used in the previous i.g. study (see section 2.10.1) for two reasons. Firstly, it had been observed in the previous *in vivo* study that curcumin was forming a solid mass in the stomach and gut of the rat. It was considered that using a suspension would improve the absorption of curcumin from the alimentary tract. Secondly, DMSO is associated with some toxicity [Brown *et al.*, 1963]. Control animals received the excipient mixture only. Tissue samples were collected 1 hr post-dose, a time point at which in preliminary experiments curcumin levels had been found to be maximal (see section 2.10.1). For an optimal comparison of this administration mode with dietary curcumin, the feeding habits of rats were exploited. Rodents are subject to a diurnal cycle with two eating peaks, one of which occurs at around 8:00 p.m. [Vermeulen *et al.*, 1997]. Rats were deprived of food for 6 hr commencing at 13.00. They then received the diet containing 2% curcumin for 3 hr coinciding with the eating peak. The dose as calculated by weighing food removal to be between 200 and 400 mg/kg. Subsequently food was withdrawn for 1 hr, after which blood and tissue samples were collected. This “starvation-refeeding” protocol was performed either in unpretreated animals or in rats which had received the curcumin diet continually for 7 days. At the end of the feeding period or subsequent to i.g.
administration, rats were subjected to terminal anesthesia (halothane/nitrous oxide) and blood was removed by cardiac puncture. Blood was placed in heparinised tubes, plasma was obtained by centrifugation (2800 x g, 4°C, 15 min). The small intestine, large intestine and liver were dissected out. The intestinal tissues were flushed thoroughly with PBS and the colonic mucosa cells were removed by scraping the mucosal surface. The liver was scissor minced and a 10% (w/v) tissue homogenate was prepared using ice cold PBS. The internal standard was added to the homogenate, vortexed and allowed to equilibrate on ice in the dark for 30 min. Levels of curcuminoids in liver and colon mucosa were determined using a curcumin calibration curve. In order to inactivate hepatic drug metabolising enzymes, the 10% liver homogenate used for preparation of this standard curve was maintained at 80°C for 10 min. Plasma, liver and colon were extracted with ethyl acetate: propan-2-ol and analysed using the “gradient HPLC conditions” and mass spectrometry method described in sections 2.4 and 2.5 respectively. Extraction efficiencies of curcumin and its metabolites from liver and colon mucosa were similar to those obtained for plasma.
2.10.3 Enzymatic hydrolysis of rat plasma samples

Rat plasma (100 μl) was incubated with β-glucuronidase (100,000 U/ml), aryl-sulphatase (1,000 U/ml) or HPLC grade H₂O at 37°C for one hr. Curcumin and its putative metabolites were extracted as described in section 2.4.

2.10.4 Comparison of 3 formulations of curcumin given by i.g. intubation

Female F344 rats received curcumin formulations/prodrug by i.g. intubation (100 mg/kg, vehicle: corn oil; dosage volume: 2.0 ml/kg). The following formulations/prodrug were administered: (1) curcumin GLA ester (see Figure 2.4), (2) “Phytopharm P54” (20 mg curcuminoids and 200 mg of essential oils extracted from the Curcuma Xanthorrhiza and Curcuma Domestica plants) and (3) curcumin (Sigma-Aldrich Comp. Ltd.) suspended in corn oil. The curcumin GLA ester was unstable in air and consequently was stored under nitrogen gas. The doses were calculated so that equimolar amounts of curcumin species were administered to each animal. Animals were subjected to terminal anaesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture 30 min and 3, 6 and 24 hr after dosing. Plasma was extracted with ethyl acetate; propan-2-ol and analysed using the “gradient HPLC conditions” described in section 2.4.
2.11 Metabolism of curcumin in vivo in humans

Fifteen patients enrolled in the dose escalation pilot study of "Phytopharm P54FP" based at the Leicester Royal Infirmary. The trial was approved by the local ethics committee and the administration of the formulation to patients by the UK Medicines Control Agency. Written informed consent was obtained from all patients. The patients had advanced metastatic adenocarcinoma of the colon or rectum for which no further conventional therapies were available.
Patients received curcumin in a capsule formulation, “Phytopharm P54FP”. Each 220 mg PF4FP capsule contained 20 mg curcuminoids (18 mg curcumin and 2 mg desmethoxycurcumin) and 200 mg essential oils derived from Curcuma plants (150 mg from Curcuma Xanthorrhiza and 50 mg from Curcuma Domestica). The curcuminoid content of the capsules was corroborated by HPLC/mass spectrometry. The essential oils are comprised of a mixture of constituents including tumerones, atlantone, curlone and zingiberene [Sharma et al., 2001b]. There were two reasons for use of this particular formulation in a clinical trial. Firstly, the curcumin dose is comparable to dietary intake of turmeric in the Asian subcontinent (see section 1.3). Secondly, it is conceivable that the essential oils may improve the oral bioavailability of curcumin or indeed have biological activity per se.

The patients recruited met the following criteria: histopathologically proven adenocarcinoma of the colon or rectum for which no conventional therapies were available and no previous investigational or chemotherapeutic drug within 28 days of enrolment. Exclusion criteria included: active chronic inflammatory or auto-immune disease; active infection, including viral infection; significant impairment of gastrointestinal function or absorption; active peptic ulcer disease; known biliary obstruction or biliary insufficiency, and use of non-steroidal anti-inflammatory drugs (NSAIDs) within 14 days of enrolment. Patients were asked to abstain from NSAID use and the consumption of foods containing the spice turmeric during the period of study.

The trial was a dose escalation study with three patients at each dose level. Patients received 2, 4, 6, 8 or 10 capsules once daily, which equates to 36, 72, 108, 144 and 180 mg curcumin respectively. Treatment was continued until disease progression was established or consent was withdrawn.
Blood, urine and faeces were collected on days 1, 2, 8 and 28 and monthly. All samples were wrapped in foil to protect them from light. Blood collection on day 1 of treatment was pre-dose and at 30, 60, 120, 180 and 360 min post-dose. Samples were stored in tubes pre-treated with lithium-heparin. Plasma was prepared from whole blood as described in section 2.4 and stored at -80°C until analysis. Curcuminoids were extracted from plasma and analysed by HPLC as detailed in section 2.4. In order to measure total curcuminoids, curcumin and conjugated curcumin, plasma was incubated with the deconjugating enzymes, β-glucuronidase and aryl sulphatase essentially as described previously [Inano et al., 2000] but with some modifications. Plasma (1 ml) was incubated with 0.01 M phosphate buffer (pH 4.6) containing 20% ascorbic acid, 0.17% EDTA, 1,250 U/ml β-glucuronidase and 25 U/ml sulphatase at 37°C for 60 min. Samples were extracted with ethyl acetate: propanol-2-ol (9:1, v/v) and analysed using the “gradient HPLC conditions” described in section 2.4.

In order to determine whether curcumin was sequestered into lipoproteins, blood (approximately 20 ml) was taken from patients at the Leicester Royal Infirmary (Leicester, UK). Plasma (approximately 12 ml) was prepared from whole blood as described in section 2.4 and stored on ice. Sodium bromide (5.45 g) was added to each plasma sample and thoroughly mixed [Schroeter, personal communication]. The plasma was layered beneath an equivalent volume of saturated saline and ultra-centrifuged (100,000 x g, 4°C, 120 min). Approximately 1 ml of total lipoproteins was obtained using this method. The lipoproteins were acidified with 200 µl TCA in order to precipitate the proteins. Curcumin was extracted from the total lipoproteins with an equivalent volume of ethyl acetate. This method did not facilitate detection of curcumin sulphate in the plasma, due to cleavage of the sulphate moiety under the acidic assay conditions. The solvent was removed by evaporation under a stream of
nitrogen gas. The residue was reconstituted in acetonitrile: water (1:1, v/v) and analysed by HPLC as detailed in section 2.4.

Red blood cells (approximately 1 ml) were extracted twice with ethyl acetate (twice volume of sample) and mixtures were centrifuged (2,800 × g, 4°C, 15 min). The organic layers were removed, combined and evaporated to dryness under nitrogen. Samples were reconstituted in acetonitrile: water (1:1, v/v) and analysed by HPLC as detailed in section 2.4.

White blood cells were separated from other blood cells by addition of 10 ml PBS to fresh blood (10 ml) and mixed. The blood was layered over Ficoll-paque (10 ml) and centrifuged (400 × g, 21°C, 30 min). The middle layer containing white blood cells was removed and acidified by addition of 1 M acetate buffer (2 ml). The white blood cells were extracted twice with an equal volume of ethyl acetate and centrifuged (2800 × g, 4°C, 15 min). The organic layers were removed, combined and evaporated to dryness under nitrogen. Samples were reconstituted in acetonitrile: water (1:1, v/v) and analysed by HPLC as detailed in section 2.4.

Urine samples were allowed to defrost at room temperature in the dark. The samples were adjusted to pH 4.6 with concentrated acetic acid. The urine was extracted twice with 20 ml of ethyl acetate: propan-2-ol (9:1, v/v). The organic layers were combined and dried under a stream of nitrogen gas. The samples were stored at -80°C in preparation for HPLC analysis. Samples were reconstituted in acetonitrile: water (1:1, v/v) and analysed by HPLC as detailed in section 2.4.

Curcumin and curcumin sulphate were extracted from faeces with two parts (w/v) of acetonitrile: water (3:2, v/v). Curcuminoids were separated from other faecal constituents by
Chapter 2

C18 solid phase extraction. The column was primed with 2 ml of acetonitrile, followed by 5 ml of H$_2$O. Samples were diluted with four parts water (v/v) and washed through the column with H$_2$O (5 ml). Curcumin and curcumin sulphate were eluted from the column with acetonitrile (2 ml) and analysed by HPLC and LC-MS as described in sections 2.4 and 2.5 respectively. Neither curcumin nor its metabolites, curcumin sulphate or curcumin glucuronide, were detected in the water eluent. Quantification of the curcumin and curcumin sulphate in faeces was achieved using a curcumin calibration curve.

A phase I clinical study was designed and conducted by Dr. D. Brenner (University of Michigan Medical School, Michigan, US) in which the pharmacokinetics and safety of microcrystalline formulations were investigated in healthy male and female volunteers. The first microcrystalline formulation was a flavoured beverage and the second was a gelatin capsule. The study was approved by the institutional review board to the University of Michigan. Written informed consent was obtained from all participants. There were six subjects at each dose level (50, 100 and 200 mg/day) and one half of the participants received each of the two formulations. Patients were transferred to the alternative formulation after a two week ‘wash-out’ period. Participants received the single dose with a standardised meal and 10 ml blood samples were taken for pharmacokinetic studies at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 hours. Blood samples were centrifuged (2800 × g, 4°C, 15 min) and the plasma was transferred to the UK on dry ice. Curcuminoids were extracted from plasma and analysed by HPLC and LC-MS as detailed in sections 2.4 and 2.5 respectively.
3 Metabolism of curcumin by the intestine and liver
3.1 Introduction

Several metabolites of curcumin have been identified after administration of the agent in vivo (see section 1.3.4). Many drug metabolising enzymes, for example UDP-glucuronosyl transferases [Hartiala, 1973; Caldwell and Varwell, 1982], glutathione-S-transferases [de Bruin et al., 2000] and sulphotransferases [Bostrom et al., 1968] have been shown to be expressed in the human gastrointestinal tract. In vitro studies have indicated that curcumin may undergo biotransformation in the gastrointestinal tract [Ravindranath and Chandrasekhara, 1981]. When [3H] radiolabelled curcumin was incubated with everted rat gut sacs, it was found to be biotransformed during its passage across the gut wall [Ravindranath and Chandrasekhara, 1981]. In a recent study, curcumin was shown to be rapidly metabolised by rat gut homogenate, although its metabolites were only ambiguously identified [Asai and Miyazawa, 2000]. In order to investigate whether curcumin undergoes metabolic biotransformation in the gastrointestinal tract, the agent was incubated with gut subcellular fractions. Based on the available knowledge of curcumin metabolism (see section 1.3.4), three metabolic routes were studied in vitro: sulphation, glucuronidation and reduction. The liver has a major role in detoxification of xenobiotic compounds. Therefore to establish the quantitative significance of the gut to the overall metabolism of curcumin, hepatic and intestinal curcumin metabolism were compared. Thus far, curcumin metabolism has been studied primarily in the rat [Ravindranath and Chandrasekhara, 1980; Ravindranath and Chandrasekhara, 1981; Wahlstrom and Blenow, 1978; Holder et al., 1978]. To validate the use of the rat as a model for humans, curcumin metabolism was compared between human and rat tissues.
Subcellular fractions, which are often used in metabolism studies are considered artificial systems due to the loss of cellular integrity and high concentrations of cofactors added to the incubations. In order to obtain a more realistic picture of curcumin metabolism in vivo, the metabolism of this agent was investigated in intact rat gut and in suspensions of isolated human and rat hepatocytes.

### 3.2 Metabolism of curcumin by subcellular fractions

Curcumin has been shown to undergo metabolic conjugation and reduction in vivo (see section 1.3.4). In order to study curcumin metabolism in vitro, the agent was incubated with cytosol or microsomes prepared from human or rat liver and intestine. Appropriate cofactors were added to the incubations. Curcumin and its putative metabolites were identified by reversed phase HPLC with mass spectrometry in the selected ion mode or UV-visible detection and co-chromatography with authentic standards (see section 2.3). The gradient HPLC method described in section 2.4 was used for this analysis and the retention times of curcumin and its putative metabolites are summarised in Table 3.1. Curcumin, curcumin sulphate and curcumin glucuronide were analysed at 420 nm and the reduced metabolites, HHC, HHCOH and THC were analysed at 280 nm.

Curcumin was biotransformed to THC and HHC by both hepatic (see Figure 3.1) and intestinal cytosol (see Figure 3.2) obtained from rats and humans. THC gave rise to a broad non-symmetrical peak (see Figure 3.2, peak 3). Whilst the tautomeric equilibration of the ketone and alcohol functions in curcumin is conceivably fast, in the case of THC (see Figure 3.2A) equilibration involving the ketone group in position 3 and the alcohol group in position 5 may be so slow that the HPLC column distinguishes the tautomers as separate species. The
metabolites were characterised using LC-MS in the selected ion mode, by molecular ions of m/z at 371 and 373 for THC and HHC respectively. A small peak that co-eluted with authentic HHCOH was also identified in the cytosolic extracts, although it was not possible to corroborate this observation by mass spectrometry. Hepatic and intestinal microsomes did not generate detectable levels of THC, HHC or HHCOH.

A peak that co-eluted with authentic curcumin sulphate was identified in human and rat hepatic (see Figure 3.3) and intestinal (see Figure 3.4) cytosolic extracts. This peak was authenticated as curcumin sulphate by its molecular ion of m/z at 447 using LC-MS. Hepatic and intestinal microsomes did not generate sufficient curcumin sulphate for detection.

HPLC analysis of extracts of incubates of curcumin with hepatic (see Figure 3.5) and intestinal (see Figure 3.6) microsomes revealed a peak that co-eluted with authentic curcumin glucuronide. This observation was corroborated by identification of a molecular ion of m/z at 543.
### Table 3.1. Retention times of curcumin and its putative metabolites using the gradient HPLC method described in section 2.4. A linear gradient of 5-45% acetonitrile in 0.01 M ammonium acetate (pH 4.6) for 30 min was used, followed by an increase over 20 min to 95% organic component. The flow rate used was 1 ml/min and the injection volume was 100 μl. Curcumin, curcumin sulphate and curcumin glucuronide were analysed at 420 nm and the reduced metabolites, HHC, HHCOH and THC were analysed at 280 nm.

<table>
<thead>
<tr>
<th>Curcuminoid</th>
<th>Retention time ± 0.5 min</th>
</tr>
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<tbody>
<tr>
<td>Hexahydrocurcuminol</td>
<td>22</td>
</tr>
<tr>
<td>Hexahydrocurcumin</td>
<td>24</td>
</tr>
<tr>
<td>Tetrahydrocurcumin</td>
<td>29-33</td>
</tr>
<tr>
<td>Curcumin sulphate</td>
<td>31</td>
</tr>
<tr>
<td>Curcumin glucuronide</td>
<td>25</td>
</tr>
<tr>
<td>Curcumin</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 3.1. Metabolism of curcumin by human (A) and rat (B) hepatic cytosol. Curcumin (100 μM) was incubated with cytosol (1 mg cytosolic protein per ml) and NADPH (10 mM) in phosphate buffer (0.01 M, pH 7.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) hepatic cytosol was incubated with vehicle (DMSO) and NADPH only. Curcumin, THC, mTHPC and HHC were detected at 280 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as HHC (2), THC (3), curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peak marked with an asterix (*) was an endogenous peak detected in control samples.
Figure 3.2. Metabolism of curcumin by human (A) and rat (B) intestinal cytosol. Curcumin (100 μM) was incubated with cytosol (1 mg cytosolic protein per ml) and NADPH (10 mM) in phosphate buffer (0.01 M, pH 7.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) intestinal cytosol was incubated with vehicle (DMSO) and NADPH only. Curcumin, THC, mTHPC and HHC were detected at 280 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as HHC (2), THC (3), curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peak marked with an asterix (*) was an endogenous peak detected in control samples.
Figure 3.2A Chemical structures of tautomers of THC
Figure 3.3. Metabolism of curcumin by human (A) and rat (B) hepatic cytosol. Curcumin (100 μM) was incubated with hepatic cytosol (1 mg cytosolic protein per ml) and PAPS (0.4 mM) in phosphate buffer (0.01 M, pH 8.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) hepatic cytosol was incubated with vehicle (DMSO) and PAPS only. Curcumin, mTHPC and curcumin sulphate were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin sulphate (5), curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The small peak marked with an asterix (*) was identified by LC-MS as desmethoxycurcumin sulphate.
Figure 3.4. Metabolism of curcumin by human (A) and rat (B) intestinal cytosol. Curcumin (100 μM) was incubated with intestinal cytosol (1 mg cytosolic protein per ml) and PAPS (0.4 mM) in phosphate buffer (0.01 M, pH 8.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) intestinal cytosol was incubated with vehicle (DMSO) and PAPS only. Curcumin, mTHPC and curcumin sulphate were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin sulphate (5) and curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peak indicated with an asterix (*) was identified by LC-MS as desmethoxycurcumin sulphate.
Figure 3.5. Metabolism of curcumin by human (A) and rat (B) hepatic microsomes. Curcumin (100 µM) was incubated with hepatic microsomes (1 mg microsomal protein per ml), UDPGA (3 mM), magnesium chloride (50 mM) and Triton X-100 (0.01%) in phosphate buffer (0.01 M, pH 7.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) hepatic microsomes were incubated with vehicle (DMSO) and cofactors only. Curcumin, curcumin glucuronide and mTHPC were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin glucuronide (4), curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peak indicated with an asterix (*) was identified by LC-MS as desmethoxycurcumin glucuronide.
Figure 3.6. Metabolism of curcumin by human (A) and rat (B) intestinal microsomes. Curcumin (100 μM) was incubated with intestinal microsomes (1 mg microsomal protein per ml), UDPGA (3 mM), magnesium chloride (50 mM) and Triton X-100 (0.01%) in phosphate buffer (0.01 M, pH 7.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, human (a) or rat (b) intestinal microsomes were incubated with vehicle (DMSO) and cofactors only. Curcumin, mTHPC and curcumin glucuronide were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin glucuronide (4) and curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are due to desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peak indicated with an asterix (*) was identified by LC-MS as desmethoxycurcumin glucuronide.
3.2.1 Quantification of curcumin metabolites

Quantification of curcumin and its metabolites was achieved using a suitable calibration curve and internal standard. The amounts of curcumin metabolites generated by the human and rat subcellular fractions are shown in Table 3.2. These values should only be considered as semi-quantitative as the poor extraction efficiency of these metabolites decreased the accuracy of the values. The values were thought to allow useful comparison of curcumin metabolism between tissues and species. It was not possible to quantify THC formation due to the broad shape of its chromatographic peak (see Figure 3.2, peak 3).

There was considerable inter-species variation in the metabolism of curcumin. Subcellular fractions derived from human intestine generated more curcumin sulphate (see Figure 3.4), curcumin glucuronide (see Figure 3.6) and HHC (see Figure 3.2) than the fractions prepared from the equivalent rat tissue (see Table 3.2). The human intestinal cytosolic cell fraction generated four times more curcumin sulphate and eighteen times more HHC than rat intestinal cytosol. Microsomal intestinal metabolism of curcumin yielded sixteen times more curcumin glucuronide in humans than in the equivalent fraction in the rat. In liver, cytosolic reduction of curcumin to HHC was seven times more abundant in humans relative to the corresponding tissue in rats (see Figure 3.1). Rat hepatic cytosol produced five times more curcumin sulphate than cytosol prepared from human liver (see Figure 3.3). Rat liver microsomes generated ten times more curcumin glucuronide conjugate than human hepatic microsomes (see Figure 3.5). The difference in these values was found to be significant using analysis of variance (ANOVA) (see section 2.6.4) (p> 0.05).
The reduction of curcumin to HHC occurred to a similar extent in human hepatic (see Figure 3.1A) and intestinal cytosol (see Figure 3.2A). There was three times more HHC formed in rat liver (see Figure 3.1B) than intestine (see Figure 3.2B), although this difference did not reach significance. In humans, formation of curcumin glucuronide in the intestine (see Figure 3.6A) was approximately twice that observed in the liver (see Figure 3.5A), although this difference was not significant. In the rat, the amount of curcumin glucuronide formed in the intestine (see Figure 3.6B) was significantly (p<0.05) lower than that measured in the liver (see Figure 3.5B). Curcumin sulphation was three-fold higher in cytosol from human intestine (see Figure 3.4A) than in that from human liver (see Figure 3.3A), whereas in the rat, intestinal sulphation (Figure 3.4B) was only a seventh of that in the liver (see Figure 3.3B). These differences in the formation of curcumin sulphate were significant (p>0.05).
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intestine</th>
<th>Liver</th>
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<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Microsomes</td>
</tr>
<tr>
<td></td>
<td>Rat&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Human&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HHC</td>
<td>8 ± 3</td>
<td>142 ± 92</td>
</tr>
<tr>
<td>CS</td>
<td>25 ± 3</td>
<td>103 ± 23</td>
</tr>
<tr>
<td>CG</td>
<td>N.D.</td>
<td>N.D.</td>
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</tbody>
</table>

Table 3.2. Amounts of curcumin metabolites (nmol per mg of protein) following incubation of hepatic and intestinal cytosolic and microsomal fractions prepared from humans and rats with curcumin (see 2.6.4) for 60 min. Curcumin and its metabolites were analysed by HPLC with UV-visible detection (see section 2.4). Curcumin sulphate and curcumin glucuronide were detected at 420 nm, whereas HHC was analysed at 280 nm. The amounts of curcumin sulphate and curcumin glucuronide generated by human intestinal cytosol and microsomes respectively that are shown here are lower than those that would be predicted from preliminary studies (see section 2.6.4). However, these values are still significantly different to amounts of curcumin sulphate and curcumin glucuronide generated by rat intestine and human liver. <sup>a</sup> Mean ± SD (n = 4), <sup>b</sup> Mean ± SD (n = 3), N.D. = Not detected, CS=Curcumin sulphate, CG=Curcumin glucuronide.
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It has been suggested that there is a decrease in the activity of UDP-glucuronosyl transferases [Hanninen and Aitio, 1968] and sulphotransferases [Schwarz and Schwenk, 1984] from the upper to lower gastrointestinal tract. The decrease in the activity of these enzymes towards the aboral section of the gut is thought to be partly due to increasing activity of microbial proteinases [Hanninen et al., 1987]. In order to determine whether there is a difference in curcumin metabolism between different sections of the gut, curcumin was incubated with subcellular fractions derived from rat jejunum or colon. Cytosol prepared from the jejunum and colon generated 25 ± 3 and 40 ± 9 nmol of curcumin sulphate per mg of protein in 60 min (mean ± SD, n = 3) respectively. The amount of HHC generated by colonic cytosol was below the limit of quantification, whereas 8 ± 3 nmol of HHC was produced by cytosol prepared from the jejunum. Microsomes prepared from jejunum or colon generated 16 ± 1 and 42 ± 11 nmoles curcumin glucuronide per mg protein, respectively (mean ± SD, n=3). The differences in the amount of curcumin metabolites generated by jejunal and colonic cytosol were not found to be significant.

3.3 Metabolism of curcumin by intact rat gut

In order to explore whether the curcumin metabolites formed by gut fractions in vitro (see section 3.2) are also generated in intact intestinal tissue, the agent was incubated with everted rat gut sacs. Gut sacs were maintained in buffer at 37°C under a continual stream of carbogen. Mucosal media, serosal media and jejunal tissue were analysed by HPLC with UV/visible detection at 280 and 420 nm. Curcumin was identified in mucosal media 30, 60 and 180 min (see Figure 3.7A) after addition of curcumin. Curcumin was also detectable in the jejunal tissue and serosal media 180 min after commencement of the incubation. Peaks
that co-eluted with authentic curcumin sulphate and curcumin glucuronide were detected in the mucosal media 60 min after addition of curcumin (result not shown), but not at any other time points. The peak that co-eluted with curcumin sulphate was authenticated as curcumin sulphate by LC-MS (see Figure 3.8). The peak assigned as curcumin glucuronide by co-chromatography eluded identification by LC-MS. The reduction products of curcumin, HHCOH, HHC and THC, were not detected.
Figure 3.7. Metabolism of curcumin by everted gut sacs (A). Curcumin (100 μM) was incubated with gut sacs in Kreb’s Ringer phosphate buffer (pH 7.4) and maintained at 37°C for 180 min. Curcuminoids were extracted with ethyl acetate: propan-2-ol (9:1, v/v). Chromatogram (a) represents incubation of curcumin (100 μM) with buffer only. Curcuminoids were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin (6), desmethoxycurcumin (7) and bisdesmethoxycurcumin (8).
3.4 Metabolism of curcumin by isolated rat and human hepatocytes

3.4.1 Identification of metabolites

Hepatocytes make up approximately 80% of the human liver by weight and 93% by volume. Studies by Wahlstrom and Blenow [1978] indicated that curcumin undergoes rapid metabolic removal in suspensions of isolated rat hepatocytes, although its metabolites were not identified. Hepatocytes were isolated from either male rats or human livers and incubated with curcumin. The extracts of cells and medium were analysed by HPLC. The pattern of curcumin metabolism in rat and human hepatocytes was qualitatively similar. Curcumin and two putative metabolites were detected at 280 nm (see Figure 3.9), whereas only a peak co-eluting with curcumin was detected at 420 nm (see Figure 3.10). Metabolites 1 and 2 (see Figure 3.9) had retention times of 22 and 24 min respectively. Metabolite 2 was found to co-
elute with authentic HHC. Mass spectral analysis revealed the molecular ion of metabolite 2 to be m/z at 373 (see Table 3.3). Further characterisation of metabolite 2 was possible by collection of the fraction that corresponded to this metabolite and concentration of the sample by lyophilisation. Subsequent analysis by mass spectrometry revealed a similar fragmentation pattern for authentic HHC and metabolite 2 (see Table 3.3).

Mass spectral analysis in the negative ion mode demonstrated that the molecular ion of metabolite 1 was m/z at 375 (see Table 3.3), therefore two mass units larger than HHC. It was postulated that metabolite 1 might be a further reduction product of HHC, hexahydrocurcuminol (HHCOH). Authentic HHC was incubated at room temperature with the reducing agent sodium borohydride. The ethyl acetate extract of this reaction was analysed by HPLC (absorbance at 280 nm) and LC-MS in the selected ion mode (result not shown). The reaction product was found to have a molecular ion of m/z at 375; a retention time of 22 min and a fragmentation pattern similar to that of metabolite 1 (see Table 3.3). To confirm that HHCOH was a hepatocytic reduction product of HHC, the compound was incubated with isolated hepatocytes. Analysis of extracts of the incubates by HPLC revealed a peak that co-eluted with metabolite 1, which was consistent with the identity of HHCOH (see Figure 3.11).

Figure 3.12 shows a time course of disappearance of curcumin together with the concurrent generation of HHC and HHCOH in suspensions of rat hepatocytes. These data demonstrate that the reduction product HHC is generated rapidly from curcumin. The ratio of integrated peak areas of HHC over HHCOH after incubation of rat or human hepatocytes with curcumin for 2 hr was 1.0 ± 0.1 in the case of rat hepatocytes compared with 3.2 ± 0.6 (mean ± SD, n = 3 for each) for human hepatocytes. Therefore the overall reduction of curcumin to HHCOH,
the ultimate reduction step, occurs more extensively in hepatocytes isolated from rats than those derived from humans.
Table 3.3. Characterisation of curcumin and its putative metabolites by HPLC and mass spectrometry. *m/z values of the most prominent product ions in the mass spectrum. The percentage abundance of each product ion is in parentheses. **Molecular ion.
Figure 3.9. Metabolism of curcumin by suspensions of isolated human (A) or rat (B) hepatocytes. Curcumin (100 μM) was incubated with isolated hepatocytes (2×10⁶ cells/ml) in liver suspension buffer. Incubates were maintained at 37°C for 90 min and extracted with ethyl acetate. In control experiments, human (a) or rat (b) isolated hepatocytes were incubated with vehicle (DMSO) only. Curcumin, HHC and HHCOH were detected at 280 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as HHCOH (1), HHC (2), curcumin (6) and mTHPC (9). The two small peaks beyond curcumin are consistent with the presence of desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2). The peaks marked with an asterix (*) were identified in all incubates by HPLC, but eluded identification by mass spectrometry.
Figure 3.10. Metabolism of curcumin by suspensions of isolated human hepatocytes. Human hepatocytes (2 x 10^6 cells/ml) were incubated with vehicle only (A) or curcumin (B) in liver suspension buffer. The concentration of substrate used was 100 μM. Incubates were maintained at 37°C for 90 min and extracted with ethyl acetate. Curcumin was detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Curcumin (6) and mTHPC (9) were identified by co-chromatography and LC-MS. The two small peaks beyond curcumin are consistent with the presence of desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2).
Figure 3.11. Metabolism of HHC by suspensions of isolated human hepatocytes. HHC was incubated with liver suspension buffer only (A) or hepatocytes ($2 \times 10^6$ cells/ml) (B). The concentration of substrate used was 100 $\mu$M. Incubates were maintained at 37°C for 90 min and extracted with ethyl acetate. HHC and HHCOH were detected at 280 nm and gradient HPLC conditions were as described in section 2.4. HHCOH (1) and HHC (2) were identified by co-chromatography and LC-MS.
Figure 3.12. Time course of disappearance of parent compound, curcumin (A) and appearance of the reduced metabolites, HHC (B) and HHCOH (C). Curcumin (100 μM) was incubated with isolated rat hepatocytes for 5, 15, 30, 60 or 90 min and extracted with ethyl acetate. Curcumin and its metabolites were analysed by HPLC as described in section 2.4 with detection at 280 nm. The values calculated were the mean ± SD following preparation of hepatocytes from three separate animals.
3.4.2 Conjugation of curcumin by isolated hepatocytes

HPLC analysis of extracts of incubates of curcumin with isolated human hepatocytes with detection at 420 nm revealed only one major peak, that of parent compound (see Figure 3.10.). Peaks that co-eluted with curcumin sulphate and curcumin glucuronide were near the detection limit. Two assays were used to determine the ability of rat and human hepatocytes to conjugate a model substrate by glucuronidation. The first assay was based on the formation of a diazonium salt from 2-aminophenol glucuronide (see Figure 3.13). The ultimate reaction product (an azo compound) can be analysed spectrophotometrically. The reaction is specific for 2-aminophenyl glucuronide because excess substrate (2-aminophenol) is destroyed in the acidic reaction conditions. The second assay was based on the loss of fluorescence of 7-hydroxycoumarin. As a positive control, liver microsomes and appropriate cofactors were also incubated with each substrate. Suspension of rat or human hepatocytes were unable to conjugate either of these model substrates.

\[
\begin{align*}
2\text{-aminophenyl glucuronide} & \xrightarrow{\text{Cold sodium nitrite}} \text{Diazonium salt} \\
\text{Diazonium salt} & \xrightarrow{\text{N-naphthylethylene diamine}} \text{Azo compound}
\end{align*}
\]

Figure 3.13. Assay for glucuronosyl transferase activity.
3.5 Metabolism of HHC by hepatic microsomes

HHC and HHCOH were identified as the major reduction products of curcumin following incubation of parent compound with suspensions of isolated rat and human hepatocytes, as detailed in section 3.4. In contrast, when human and rat hepatic cytosol was incubated with curcumin, THC and HHC were determined to be the predominant curcumin reduction products and HHCOH was found at levels which were near the limit of detection (see section 3.2). To explain why HHC was the major cytosolic curcumin reduction product, whereas isolated rat and human hepatocytes were able to catalyse a further reduction step to generate HHCOH, curcumin and HHC were incubated with hepatic microsomes (see Figure 3.14). Although curcumin was not a substrate for hepatic microsomes HHC was reduced to HHCOH by hepatic microsomes.
Figure 3.14. Metabolism of HHC (A) and curcumin (B) by hepatic microsomes. Liver microsomes (1 mg microsomal protein per ml) were incubated with HHC (A) or curcumin (B) and NADPH (10 mM) in phosphate buffer (0.01 M, pH 7.4). The concentration of substrate used was 100 µM. Incubates were maintained at 37°C for 90 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). Detection of HHCOH, HHC and curcumin was achieved at 280 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as HHCOH (1), HHC (2) and curcumin (6). The two small peaks beyond curcumin are consistent with the presence of desmethoxycurcumin and bisdesmethoxycurcumin (see section 2.2).
3.6 Discussion

The *in vitro* hepatic and intestinal metabolism of curcumin was investigated. Previous studies have demonstrated that rat liver [Wahlstrom and Blenow, 1978; Asai and Miyazawa, 2000] and intestine [Asai and Miyazawa, 2000] have a role in curcumin metabolism, although the metabolites have not been identified. The data presented in this chapter are the first to allow unambiguous identification of *in vitro* hepatic and intestinal metabolites of curcumin in both rats and humans. In order to investigate the glucuronidation, reduction and sulphation of curcumin, three experimental systems were used: subcellular fractions, suspensions of isolated hepatocytes and intact rat intestine.

The data presented in this chapter highlights the role of the intestinal tract in the biotransformation of curcumin, particularly in humans. In order to determine the relative importance of the gut in curcumin metabolism, glucuronidation, sulphation and reduction were compared between intestinal and hepatic subcellular fractions. In humans, more curcumin glucuronide and curcumin sulphate were generated by subcellular fractions prepared from intestine than liver. Extensive metabolism of curcumin by the intestine may contribute to the poor bioavailability of this agent. Indeed rapid metabolism by gut drug metabolising enzymes is thought to account for the poor bioavailability of other drugs, for example the oral contraceptive ethinyloestradiol [Back and Rogers, 1987].

The rat has been used as a model of curcumin metabolism in humans [Wahlstrom and Blenow, 1978]. In order to determine the validity of this model for prediction of curcumin metabolism in humans, curcumin conjugation and reduction were compared between rat and human tissues. Conjugation and reduction of curcumin was found to be qualitatively similar
in rat and human tissues. However, quantitative analysis revealed that the human intestinal
cytosolic fraction generated more curcumin sulphate, curcumin glucuronide and HHC than
the equivalent tissue derived from the rat. In contrast, rat hepatic subcellular fractions yielded
more curcumin metabolites than subcellular fractions prepared from human liver. Thus, the
rat may not be a suitable model for the study of quantitative aspects of curcumin conjugation
and reduction. The results suggest that experiments in the rat may underestimate the extent of
intestinal metabolism that occurs in humans. Indeed previous studies have suggested that
there is little extrahepatic expression of sulphotransferases in the rat [Eisenhofer et al., 1999].

In order to investigate whether the metabolites generated \textit{in vitro} by subcellular fractions were
also generated by intact rat gut, curcumin was incubated with everted rat gut sacs. Work by
Ravindranath \textit{et al.} demonstrated that $[^3\text{H}]$-curcumin was biotransformed by this tissue to
generate a product with increased hydrophilicity, although this metabolite was not identified
[Ravindranath and Chandrasekhara, 1981]. Following incubation of curcumin with everted
rat gut sacs, curcumin sulphate was the only metabolite to be incontrovertibly identified in the
mucosal medium. Interestingly, curcumin sulphate could only be detected in the medium 60
min after commencement of the incubation. When curcumin was incubated with everted rat
gut sacs for 180 min, the peak area of curcumin and desmethoxycurcumin (Figure 3.7A) was
decreased compared to the control (Figure 3.7a). In contrast, the peak area of
bisdesmethoxycurcumin remained constant. Bisdesmethoxycurcumin was found to be a poor
substrate of sulphotransferases (see Figure 3.4). It would seem sensible to propose that this is
due to lack of compatibility between bisdesmethoxycurcumin and the active site of the
sulphotransferase enzyme. Therefore by indirect conclusion, one may postulate that extensive
metabolic removal of curcumin by sulphation is taking place.
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Curcumin has been shown previously to be rapidly metabolised by isolated rat hepatocytes [Wahlstrom and Blenow, 1978], although its metabolites were not identified. The data presented in section 3.4.1 demonstrates for the first time that curcumin is metabolised to HHC and HHCOH by human and rat hepatocytes. The metabolism of curcumin was qualitatively similar in rat and human hepatocytes. THC and conjugates of the reduced metabolites were found to be below the limit of detection. The glucuronide and sulphate conjugates of the parent compound were only minor metabolites. The rat and human hepatocytes used in this work did not conjugate model substrates by glucuronidation. The lack of conjugative ability in these rat and human hepatocytes is in contrast to the results of other investigators [Fry et al., 1979; McPhail et al., 1993].

The cellular fraction involved in the generation of HHC and HHCOH was investigated. The source of the enzyme that reduced curcumin to generate HHC was exclusively in the cytosolic fraction, whilst the biotransformation of HHC to HHCOH (see Figure 3.15) was catalysed by both cytosolic and microsomal enzymes. The differential subcellular compartmentalisation with respect to curcumin reduction may help explain why its complete reduction to HHCOH seems to occur more efficiently in the intact human and rat hepatocyte than in human and rat hepatic cytosol. A possible candidate enzyme responsible for the catalytic generation of HHCOH from HHC in microsomes is NADPH cytochrome P450 reductase, although this has not been experimentally confirmed.
The fact that the extent of metabolism of curcumin in vitro varied according to the system used highlights the pertinence of using several systems to attempt prediction of the in vivo metabolism of a compound. For example, curcumin reduction products were not measured in the intact rat gut tissue, but rat gut cytosol was able to catalyse the reduction of curcumin to HHC. Subcellular fractions often have a higher metabolic capacity than the cells or tissues from which they arise. This may be a consequence of poor cell uptake and cofactor limitation in the intact tissue. In addition, the curcumin conjugates, curcumin glucuronide and curcumin sulphate were generated by hepatic cytosol, but not by isolated hepatocytes. The reason for this difference is not clear, but it reinforces the argument that in vitro metabolic studies are only useful to give an indication of a compounds metabolism in vivo.
4 Enzymes involved in the biotransformation of curcumin to curcumin sulphate and hexahydrocurcumin
4.1 Introduction

Curcumin is metabolised by intestinal and hepatic enzymes. The reduction products, THC, HHC and HHCOH, and the phase II metabolites, curcumin sulphate and curcumin glucuronide, have been identified in vitro (see sections 3.2 and 3.4), but details of the enzymes involved in these biotransformations have hitherto not been studied. The enzyme(s) involved in the metabolic generation of curcumin sulphate were investigated using recombinant phenol sulphotransferases. In addition the sulphation of curcumin by colonic cell line cytosol was investigated. The enzymatic reduction of curcumin to HHC was studied using baker’s yeast and isolated alcohol dehydrogenases.

4.2 Sulphation of curcumin

Six isoenzymes of the human phenol sulphotransferase subfamily are expressed in the human gut [Eisenhofer et al., 1999]. To determine whether two of these enzymes might be involved in the sulphation of curcumin, recombinant SULT1A1 and SULT1A3 were incubated with curcumin in the presence of PAPS for 60 min. SULT1A1 and SULT1A3 generated 6 and 24 nmol/µg of curcumin sulphate respectively (see Figure 4.1). Curcumin sulphate (m/z at 447) was identified as the reaction product by mass spectrometry in the selected ion mode (data not shown). Immunoblot analysis confirmed that SULT1A1 and 1A3 are expressed in human hepatic and intestinal tissue (see Figure 4.2).
Figure 4.1. Metabolism of curcumin by recombinant SULT1A3 (A) and SULT1A1 (B). SULT1A1 and SULT1A3 (10 μg/ml) were incubated with curcumin (100 μM) and PAPS (0.4 mM) in phosphate buffer (0.01 M, pH 8.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, SULT1A3 (a) or SULT1A1 (b) were incubated with vehicle (DMSO) and cofactors only. The experiment was performed in triplicate and the chromatograms shown are representative of three analyses. Curcumin and curcumin sulphate were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin sulphate (5) and curcumin (6), desmethoxycurcumin (7), bisdesmethoxycurcumin (8) and mTHPC (9).
Figure 4.2. Immunoblot analyses of human intestinal and hepatic tissue samples (30 μg protein) using a monoclonal primary antibody (1:1000 dilution) against phenol sulphotransferase isoenzymes SULT1A1 and 1A3, and an anti-sheep secondary antibody (1:4000 dilution). The lanes were assigned as follows: (A) standard recombinant SULT1A3; (F) standard recombinant SULT1A1; (B), (C) and (D) jejunal cytosol from 3 individuals and (E) human liver cytosol. The SULT standards are not aligned with the lanes at the centre of the gel. This phenomenon is referred to as a “smile effect” and is a consequence of faster migration of proteins at the centre of the gel due to higher charge density.

4.3 Reduction of curcumin

The reductive products of curcumin, HHC and HHCOH, have been identified as curcumin metabolites in vitro (see section 3.4). Curcumin is reduced by the cytosolic (see Figure 3.2), but not the microsomal (see Figure 3.14) subcellular fraction. Baker’s yeast is a convenient source of non-specific reductases. Baker’s yeast is a whole cell homogenate and therefore will contain all the cellular cofactors. Curcumin was incubated with baker’s yeast and the extracted medium analysed by HPLC. Products of curcumin reduction were below the limit of detection (result not shown). Alcohol dehydrogenase (ADH) is a cytosolic reductive
enzyme that is ubiquitously expressed in intestinal and hepatic tissue. In order to study the reduction of compounds by isolated alcohol dehydrogenases, a source of NADPH is required. ADH isolated from horses and yeast was incubated with curcumin for 60 min. The reduction product of curcumin, HHC, was generated by equine ADH (see Figure 4.3A), but not by ADH purified from yeast (result not shown). THC and HHCOH were below the limit of detection.
Figure 4.3. Metabolism of curcumin by equine ADH (A). ADH (10 U/ml) was incubated with curcumin (100 μM) and NADPH (10 mM) in phosphate buffer (0.01 M, pH 7.4). In control experiments (a), curcumin (100 μM) was incubated with ADH which had been boiled for 10 min. Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). Curcumin and its metabolites were detected at 280 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as HHC (2) and curcumin (6). The experiment was performed in triplicate and the chromatograms shown are representative of three analyses. The peak marked with Y is an endogenous peak, which is present in the ADH. The peak marked with X was not detected in the control.
4.4 Metabolism of curcumin by human colonic cellular cytosol

Curcumin sulphate has been identified as a metabolite of curcumin in incubations of HCA-7 tumour cells in culture [Plummer, personal communication]. Curcumin was incubated with cytosol prepared from the HCA-7 cells and a nonmalignant colon cell line (HCEC). The incubations were fortified with the cofactor PAPS. Curcumin was metabolised to curcumin mono-sulphate by cytosol derived from both cell lines (see Figure 4.4). The tumour cell line generated ten times more curcumin sulphate than the normal cell line. Immunoblot analyses of cytosol derived from HCA-7 and HCEC cells demonstrated expression of SULT1A1 in both cell lines (see Figure 4.5). SULT1A3 was found to be expressed in the HCA-7 cytosol, but was below the limit of detection in cytosol prepared from the normal colonic cell line. Cytosol derived from two other tumour cell lines, SW 480 and HT-29, was also analysed by immunoblotting for SULT1A3 and SULT1A1. SULT1A3 and SULT1A1 were found to be expressed in both cell lines (data not shown).
Figure 4.4. Metabolism of curcumin by cytosol derived from HCA-7 (A) and HCEC (B) cells. Cytosol (1 mg protein per ml) was incubated with curcumin (100 μM) and PAPS (0.4 mM) in phosphate buffer (0.01 M, pH 8.4). Incubates were maintained at 37°C for 60 min and extracted with ethyl acetate: propan-2-ol (9:1, v/v). In control experiments, cytosol prepared from HCA-7 (a) or HCEC (b) cells was incubated with vehicle (DMSO) and PAPS only. Curcumin and its metabolites were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. The experiment was performed in triplicate and the chromatograms shown are representative of three analyses. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin sulphate (5) curcumin (6) and mTHPC (9).
Figure 4.5. Immunoblot analyses of cytosol derived from HCA-7 and HCEC cell lines (30 μg protein) using a monoclonal primary antibody (1: 1000 dilution) against phenol sulphotransferases isoenzymes SULT1A1 and 1A3 and anti-sheep secondary antibody (1:4000 dilution). The lanes were assigned as follows: (A) standard recombinant SULT1A3; (F) standard recombinant SULT1A1; (B), HCA-7 cytosol (C) HCEC cytosol (D) HCA-7 cytosol and (E) HCEC cytosol. The HCA-7 and HCEC cytosol were prepared from separate batches of cells and this blot is representative of 3 analyses.

4.5 Discussion

The results presented in this chapter allow some insight into the enzymatic reduction of curcumin to HHC and the conjugation of the agent to generate curcumin sulphate. The reduction product of curcumin, HHC, was generated by the catalytic activity of equine hepatic ADH. It is conceivable that other non-specific reductases may also contribute to curcumin reduction in vivo. The potential involvement of ADH enzymes with the cytosolic reduction of curcumin in the gut and liver raises the interesting question as to whether metabolism of curcumin may be affected by consumption of alcoholic beverages and vice versa, that reduction of alcohol may be impaired in individuals consuming large quantities of curcumin.
Curcumin sulphate was generated from curcumin by the catalytic activity of SULT1A1 and SULT1A3, two isoenzymes that are shown here to be expressed in human intestinal and hepatic tissue. Considerably more curcumin sulphate was generated by the tumour cell line, HCA-7, than the normal cell line and this may be a consequence of increased expression of SULT1A3 in that cell line (see Figure 4.5). If this increased sulphation of curcumin in the tumour compared to the normal colon cell line is representative of other tumour cell lines and in human tumours, then it is possible that more curcumin sulphate will accumulate in the tumour than in colonic normal cells. Therefore this offers a method by which tumour cells may be targeted with curcumin sulphate.
5 *In vivo* metabolism of curcumin in rodents and humans
Chapter 5

5.1 Introduction

The rat has been used as a model for humans in curcumin metabolism studies as described in section 1.3.4. Previously curcumin metabolites have been identified *in vivo* after i.v. administration of curcumin to male *Sprague-Dawley* rats [Holder *et al.*, 1978]. Glucuronide conjugates of THC and HHC were determined as the major biliary metabolites. Following commencement of this project, curcumin glucuronide has been identified as a plasma metabolite following i.g. intubation of mice [Pan *et al.*, 1999] and male *Sprague-Dawley* rats [Asai and Miyazawa, 2000]. The authors of this latter study also identified curcumin glucuronide sulphate as a plasma metabolite after a single bolus dose of curcumin [Asai and Miyazawa, 2000]. Thus far, the metabolism of curcumin has not been compared after i.v. and i.g. administration of rats. Therefore HPLC was used to quantify levels of curcumin and its putative metabolites in the plasma of rats after a single i.g. and i.v. dose of the chemopreventive agent.

The chemopreventive efficacy of curcumin has been demonstrated *in vivo* following inclusion of curcumin in the standard diet [Inano *et al.*, 1999; Inano *et al.*, 2000], whereas the pharmacokinetics have been studied following administration of a single i.g., i.v., or i.p. dose of curcumin [Holder *et al.*, 1978; Pan *et al.*, 1999]. In an attempt to compare the absorption and metabolism of curcumin following i.g. and dietary administration of the agent, an approximately equivalent dose of the agent was administered to female rats by these two routes. Following dosing of rats via these two routes, the levels of curcumin and its putative metabolites in plasma, liver and colon were compared.
It appears that curcumin is poorly absorbed from the gastrointestinal tract (see section 1.3.4). It was postulated that the bioavailability of curcumin could be improved by either using a prodrug or certain formulations of the agent. In order to investigate this hypothesis, levels of curcumin and its metabolites in the plasma of rats were compared following a single bolus dose of “Phytopharm P54” (see section 2.10.4), curcumin GLA ester (see section 2.10.4) and “sigma grade” curcumin (see section 2.2).

The ultimate aim of this project was to determine whether curcumin or products of its metabolism could be detected in blood, urine or faeces of humans following oral administration of the agent. To test this hypothesis, patients diagnosed with advanced colorectal cancer took part in a dose escalation pilot study of “Phytopharm P54FP” (see section 2.11). A phase I clinical study was designed and conducted by Dr. D. Brenner, University of Michigan Medical School (Michigan, US), in which the pharmacokinetics and safety of microcrystalline curcumin formulations were investigated in healthy male and female volunteers.

5.2 Levels of curcuminoids in plasma after i.v. and i.g. administration

Curcumin was administered to rats via the i.v. (vehicle: glycerol formal, dose: 40 mg/kg) or i.g. routes (vehicle: DMSO, dose: 500 mg/kg). Blood was removed 5, 30, 60 and 180 min after i.v. and 30 min, 1, 2, 3, 6, 12 and 24 hr after i.g. administration. Curcumin and its metabolites were extracted from the plasma and analysed by HPLC. Estimation of the amount of curcumin or putative metabolites in the plasma samples was achieved by spiking control rat plasma to produce a calibration curve. The fate of curcumin in the plasma depended on the route of administration. Following i.v. dosing of rats, plasma levels of
curcumin were found to decrease rapidly over 30 min (see Figure 5.2), and the maximum plasma concentration of curcumin attained was 4.5 µg/ml. Peaks that co-eluted with authentic curcumin glucuronide and curcumin sulphate were also detected, in addition to a peak with retention time of 18 min (see Figure 5.1). This peak was analysed by LC-MS in the selected ion mode and found to have a molecular ion of m/z at 623. The peak was thought to be due to the presence of a glucuronide sulphate conjugate of curcumin. The reduced metabolites, HHCOH, HHC and THC, could not be analysed with UV/visible detection at 280 nm because they co-eluted with peaks resulting from endogenous plasma components. LC-MS in the selected ion mode (negative ion) was used to identify the molecular ion of the following metabolites in the plasma 30 min following i.v. administration of curcumin: THC (m/z at 371), HHC (m/z at 373), HHCOH (m/z at 375), THC glucuronide (m/z at 547), HHC glucuronide (m/z at 549), curcumin sulphate (m/z at 447) and curcumin glucuronide (m/z at 543). The selected ion chromatograms of some of these metabolites are shown in Figure 5.3. The plasma of control rats which had received the vehicle only was also analysed by LC-MS, and it did not show any of the peaks described here. Following i.g. administration, curcumin was detected in the plasma 30 min, 1, 2 and 3 hr following dosing, although the level was below the limit of quantitation (data not shown). Curcumin sulphate, curcumin glucuronide and curcumin glucuronide sulphate (see Figure 5.4) were detected in the plasma of rats up to 12 hr following dosing, although the levels were below the limit of quantitation after 6 hr. The levels of curcumin sulphate and curcumin glucuronide in the plasma up to 6 hr following dosing with parent compound are shown in Figure 5.6. The identity of curcumin glucuronide sulphate (data not shown), curcumin sulphate (see Figure 5.5) and curcumin glucuronide (see Figure 5.5) was corroborated by mass spectrometry in the selected ion mode. In order to further characterise curcumin glucuronide and curcumin sulphate, the fragmentation patterns of these metabolites were compared to that of authentic standards (see Table 5.1).
reduced metabolites, HHC, HHCOH and THC, and conjugates of the reduced metabolites were below the limit of detection. In order to support the hypothesis that curcumin conjugates had been formed *in vivo*, plasma was incubated with either $\beta$-glucuronidase (see Figure 5.4) or aryl-sulphatase (data not shown). Following incubation of plasma with $\beta$-glucuronidase, the peak assigned to curcumin glucuronide disappeared with a concurrent increase in a peak that co-eluted with authentic curcumin. Similarly when plasma was incubated with aryl-sulphatase, the peak that co-eluted with curcumin sulphate decreased with a concomitant increase of the peak that corresponded with curcumin. The enzymes, $\beta$-glucuronidase and aryl-sulphatase could also be used to confirm the identity of peak labelled by a X (see Figure 5.4). When plasma was incubated with $\beta$-glucuronidase the peak with retention time 18 min disappeared and there was a concurrent increase in the peak corresponding to curcumin sulphate (see Figure 5.4). Upon incubation of plasma with aryl-sulphatase, there was a decrease in the peak that eluted at 18 min and an increase in the peak corresponding to curcumin glucuronide. It is therefore conceivable that this peak was a consequence of the presence of curcumin glucuronide sulphate. This observation was confirmed by LC-MS (result not shown).
Figure 5.1. Curcumin and its metabolites in the plasma of rats 30 min after i.v. administration of curcumin (40 mg/kg). Plasma samples were mixed with DMSO: methanol (1:4, v/v). Curcuminoids were detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Curcumin glucuronide (4), curcumin sulphate (5) and curcumin (6) were identified by co-chromatography and LC-MS. The peak marked with an asterix (*) was identified as curcumin glucuronide sulphate. HPLC analysis of control rats that had received vehicle only (glycerol formal) did not show any of the peaks shown here.
Figure 5.2. Levels of curcumin in the plasma following i.v. dosing (40 mg/kg) of female F344 rats.
Figure 5.3. LC-MS analysis in the selected ion mode of rat plasma 30 min after i.v. administration of curcumin (40 mg/kg). Plasma samples were mixed with DMSO: methanol (1:4, v/v). Mass spectrometric analysis was carried out in the negative ion mode. Chromatographic peaks were identified as (1) HHCOH (m/z at 375), (2) HHC (m/z at 373), (6) curcumin (m/z at 367) and (10) HHC glucuronide (m/z at 549). Note that the retention times are longer than those observed in other experiments because the chromatographic conditions used were slightly different. The linear gradient programme was: acetonitrile (5-45%) in 0.01 ammonium acetate (pH 4.6) for 60 min (as opposed to the 30 min described in other experiments), followed by an increase for 20 min to 95% acetonitrile. The retention times shown here are 67.1 min for curcumin, 39.5 min for HHC, 38 min for HHCOH and 24.9 min for HHC glucuronide. Selected ion chromatograms of plasma extracts of plasma from rats that had not received curcumin did not shown any of the peaks seen here. The peak marked with an asterix is a result of isotopic contribution of m/z at 373.
Table 5.1. Characterisation of curcumin metabolites by HPLC and mass spectrometry. a m/z values of the most prominent product ions in the mass spectrum. The percentage abundance of each product ion is in parentheses. Curcumin glucuronide and plasma metabolite 4 were analysed in the positive ion mode. b Molecular ion. * See Figure 5.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC Retention time (min)</th>
<th>Mass spectrometric product ions (m/z)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin glucuronide</td>
<td>25</td>
<td>177 (100); 369 (72); 545 (15)(^b)</td>
</tr>
<tr>
<td>Curcumin sulphate</td>
<td>31</td>
<td>367 (100); 217 (85); 149 (61); 447 (50)(^b)</td>
</tr>
<tr>
<td>Plasma metabolite 4*</td>
<td>25</td>
<td>545 (100)(^b); 369 (45); 177 (30)</td>
</tr>
<tr>
<td>Plasma metabolite 5*</td>
<td>31</td>
<td>447 (100)(^b); 367 (25); 217 (24); 149 (22)</td>
</tr>
</tbody>
</table>
Figure 5.4. Curcumin and its metabolites in the plasma of rats 30 min after i.g. dosing with curcumin (500 mg/kg). In control experiments (A), plasma was incubated with H₂O only. Plasma was incubated with β-glucuronidase (B) for 60 min at 37°C. Plasma samples were mixed with DMSO: methanol (1:4, v/v). Curcumin was detected at 420 nm and gradient HPLC conditions were as detailed in section 2.4. Chromatographic peaks were identified by co-chromatography and LC-MS as curcumin glucuronide (4), curcumin sulphate (5) and curcumin (6). The peak labelled X was tentatively assigned as curcumin glucuronide sulphate by virtue of its molecular ion (m/z at 623).
Figure 5.5. LC-MS analysis in the selected ion mode of plasma from rats 30 min after i.g. administration of curcumin (500 mg/kg). Plasma samples were mixed with DMSO: methanol (1:4, v/v). The LC-MS conditions were as described in section 2.5. Chromatographic peaks were identified as (5) curcumin sulphate (m/z at 447) and (4) curcumin glucuronide (m/z at 543). Selected ion chromatograms of extracted plasma from rats that had received vehicle only (DMSO) did not show any of the peaks seen here.
Figure 5.6. Levels of curcumin conjugates in the plasma following an i.g. dose of 500 mg/kg to female F344 rats. The red line represents curcumin sulphate and the blue line represents curcumin glucuronide.
5.3 Levels of curcuminoids in plasma and tissues after i.g. and oral administration

Female F344 rats received curcumin either admixed into the standard diet (2% in the standard diet for 3 hr) or by a single i.g. bolus (vehicle: glycerol formal: cremaphore: water, dose: 500 mg/kg). The dose ingested with the diet during the 3 hr feeding period, as adjudged by the amount of food removed from the hoppers, was between 200 and 400 mg/kg and therefore comparable with the i.g. dose. Two other subgroups of rats received curcumin either as a single i.g. bolus on 7 consecutive days or as a dietary constituent for 7 days. Blood, liver and colonic scrapings were obtained from the rats 1 hr post-dose or post removal of the food which contained curcumin. This time point was selected because it was found to be associated with the maximum plasma levels of curcumin in preliminary studies (see section 5.2). Curcumin and its putative metabolites were extracted from tissues and plasma and analysed by HPLC with UV/visible and MS detection. The pattern of absorption and metabolism differed according to the route of administration. When curcumin was given to rats admixed into the food, parent compound was detected in the plasma at levels near the limit of quantitation, but the metabolites, curcumin sulphate and curcumin glucuronide, were below the limit of detection (see Table 5.2). In contrast, when curcumin was administered by i.g. intubation, parent compound, curcumin sulphate, curcumin glucuronide and curcumin glucuronide sulphate were detected in the plasma (see Table 5.2) using LC-MS and HPLC.

The level of curcumin in the plasma was approximately three to five times greater after a single i.g. bolus than after “short feeding”. Repeated i.g. administration for 7 days led to an increase in plasma levels of curcumin and its conjugates as compared with single i.g.
administration. Curcumin levels were doubled, and levels of curcumin sulphate and curcumin glucuronide were approximately 3 and 11 times higher, respectively, than those seen after single i.g. administration. In contrast, plasma levels of curcumin after its consumption in the diet for a week, at the end of which rats were subjected to the same “starvation-refeeding” regime, did not differ substantially from those measured after short-term dietary consumption. Products of metabolic reduction of curcumin were below the limit of detection in the plasma following either route of administration.
### Plasma levels

<table>
<thead>
<tr>
<th>Dosing schedule</th>
<th>Curcumin</th>
<th>Curcumin glucuronide</th>
<th>Curcumin sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short feeding</td>
<td>&lt;4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Single i.g.</td>
<td>11 ± 3</td>
<td>30 ± 20</td>
<td>60 ± 45</td>
</tr>
<tr>
<td>7 day feeding</td>
<td>4 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Repeated i.g.</td>
<td>24 ± 10</td>
<td>347 ± 79</td>
<td>161 ± 72</td>
</tr>
</tbody>
</table>

Table 5.2. Levels of curcumin and curcumin conjugates in the plasma of rats which received curcumin in the diet or by the i.g. route. Tissues were collected 1 hr post dosing. Rats received curcumin as an i.g. bolus (500 mg/kg) once ('single i.g.') or daily on 7 consecutive days ('repeated i.g.'), or with the diet (2%) for 3 h after a period of starvation (6 h) once ('short feeding') or after unlimited access to curcumin containing diet for 7 days ('7 day feeding'). The dose, as calculated by weighing food removal, was between 200 and 400 mg/kg. Curcumin and its metabolites was analysed by HPLC with detection at 420 nm (see section 2.4).<sup>a</sup> Values are the mean ± SD of 3-4 animals. <sup>b</sup>Not detected.
The levels of curcumin and its metabolites in colon scrapings and liver was determined and expressed per gram of tissue (see Table 5.3). The curcumin metabolites, curcumin sulphate, curcumin glucuronide, THC, HHC and HHCOH, and their conjugates were below the limit of detection following either route of curcumin administration. Parent compound was detected in liver and colon scrapings following i.g. administration and dietary administration of curcumin (see Table 5.3) There was a large variation in levels of the agent measured in liver and colon scrapings between animals which had received the same dose. It is conceivable that the variation in curcumin levels following feeding is due to differences in food intake during the 3 hr feeding period. The variation may also be due to a sampling error in obtaining sections of colon and liver tissue. Despite the large variation between animals, it is clear that following “short feeding” and “7 day feeding” of curcumin there was an accumulation of curcumin in the gastrointestinal tract. There was 164 times more curcumin in the gut after “short feeding” than following a single i.g. dose. Similarly, there was 27 times more curcumin in the colonic scrapings after 7 days feeding than following repeated i.g. doses. Hepatic levels of curcumin were similar irrespective of the route of administration and there was little difference in levels of curcumin in the liver between single or repeated administration via the diet or gavage.
Table 5.3. Levels of curcumin in colon mucosa (A) and liver (B) of rats that received curcumin either in the diet or by the i.g. route. Tissues were collected 1 hr post dosing. Rats received curcumin as an i.g. bolus (500 mg/kg) once ('single i.g.') or daily for 7 consecutive days ('repeated i.g.'), or with the diet (2%) for 3 hr after a period (6 hr) of starvation ('short feeding'), or after a starvation-refeeding regimen following unlimited access to curcumin-containing diet for 7 days ('7 day feeding'). The dose of curcumin when given with the diet as calculated by weighing food removal was between approximately 200 and 400 mg/kg. Curcumin and its metabolites were analysed by HPLC as detailed in section 2.4 with detection at 420 nm. *Values are the mean ± SD of 3-4 animals.

<table>
<thead>
<tr>
<th>A. Colon mucosa</th>
<th>Curcumin levels (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosing schedule</td>
<td></td>
</tr>
<tr>
<td>Short feeding</td>
<td>103* ± 109</td>
</tr>
<tr>
<td>Single i.g.</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>7 day feeding</td>
<td>177 ± 152</td>
</tr>
<tr>
<td>Repeated i.g.</td>
<td>7 ± 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Liver</th>
<th>Dosing schedule</th>
<th>(ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short feeding</td>
<td>327 ± 350</td>
<td></td>
</tr>
<tr>
<td>Single i.g.</td>
<td>18 ± 4</td>
<td></td>
</tr>
<tr>
<td>7 day feeding</td>
<td>40 ± 47</td>
<td></td>
</tr>
<tr>
<td>Repeated i.g.</td>
<td>29 ± 4</td>
<td></td>
</tr>
</tbody>
</table>
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5.4 Levels of curcumin in the plasma following i.g. administration of 3 curcumin formulations

It is possible that the poor bioavailability of curcumin is a consequence of rapid intestinal and hepatic metabolism (see chapter 3) and/or poor absorption from the alimentary tract (see section 1.3.4). In an attempt to improve the oral bioavailability of the chemopreventive agent curcumin, two formulations/prodrugs were administered by gavage to F344 rats. Levels of curcumin and its metabolites were measured in the plasma following dosing. The levels of curcumin and its metabolites following dosing with the formulations/prodrugs were compared to those attained following dosing with a curcumin suspension. The first prodrug tested was curcumin esterified with GLA. It was postulated that esterification of each of the curcumin phenolic groups with a GLA group (see Figure 2.4) may decrease intestinal and hepatic conjugation of curcumin, resulting in increased levels of parent compound in the plasma. The hydrolysis of curcumin GLA ester to yield curcumin was thought to be catalysed by non-specific esterases expressed in the blood, although this has not been experimentally confirmed. The second curcumin formulation tested was "Phytopharm P54" (see section 2.11). This formulation contained curcuminoids and essential oils derived from the Curcuma Domestica and Xanthorrhiza plants. It was postulated that the essential oils may increase the absorption of curcuminoids from the alimentary tract. The third formulation used was "sigma grade" curcumin (see section 2.2) suspended in corn oil. Following administration of the three formulations to rats, curcumin was detected in plasma, but at a level below the limit of quantitation (data not shown). Neither the "Phytopharm P54" formulation or curcumin GLA ester improved the oral bioavailability of the chemopreventive agent. The reduced metabolites, THC, HHC and HHCOH, and conjugates thereof were below the limit of detection. Curcumin sulphate and curcumin glucuronide were detected in the plasma.
following administration of curcumin in corn oil, but not after administration of "Phytopharm P54" or curcumin GLA ester.
5.5 Pilot studies of oral curcumin in humans

A dose-escalation study of “Phytopharm P54FP” (see section 2.11) was conducted in patients diagnosed with advanced colorectal cancer. Patients received a daily oral dose of 36, 72, 108, 144 or 180 mg curcumin. Blood, urine and faeces were collected on days 1 (pre-dose), 2, 8 and 28. Blood was also collected before dose administration and 0.5, 1, 2, 3, 6 and 8 hr after administration of dose on day 1. Levels of curcumin and its metabolites, curcumin sulphate, curcumin glucuronide, THC, HHC and HHCOH, were measured. Curcumin and its metabolites were not detected in the urine, total blood lipoproteins, white blood cells, red blood cells or plasma of patients, despite the limit of detection being as low as 0.1 ng/ml. In order to confirm that curcumin conjugates had not been generated in vivo, plasma was incubated with the deconjugating enzymes, β-glucuronidase and aryl-sulphatase. Incubation of patient plasma with deconjugating enzymes did not generate detectable levels of curcumin. Faeces obtained from patients on days 8 and 28 of daily treatment were analysed for the presence of curcuminoids. A peak co-eluting with curcumin was detectable in the faeces of all 15 patients. The mass spectrum of this peak was characterised by the presence of the molecular ion (m/z at 367) and major fragments of authentic curcumin including its base peak (m/z at 149), confirming the identity of this peak as curcumin. Levels of curcumin in day 29 faecal samples from patients consuming 144 and 180 mg of curcumin were between 144 and 519 nmol/g dried faeces at the lower dose and between 64 and 1054 nmol/g dried faeces at the higher dose. The faeces of one of the patients at the highest dose contained a peak that co-eluted with curcumin sulphate. Mass spectral analysis of the HPLC peak by selected ion monitoring afforded the molecular ion of m/z at 447, and the fragmentation pattern was
comparable with that of the authentic reference compound, corroborating the identity of the peak as curcumin sulphate.

A phase I study of the pharmacokinetics of two microcrystalline curcumin formulations in healthy male and female volunteers was conducted by Dr. D. Brenner, University of Michigan Medical School (Michigan, US). Patients received an oral dose of 50, 100 or 200 mg of curcumin either as a flavoured beverage or gelatin capsule. Blood samples were taken before dose administration and 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 hr after dose administration. Curcumin and its metabolites could not be unambiguously identified in the plasma of any of the volunteers using the HPLC method described in section 2.4 or in a separate laboratory using different methods [Brenner et al., 2001]. Analysis by mass spectrometry also failed to identify curcumin or its metabolites.

5.6 Discussion

The fate of curcumin was investigated in the rat after a single i.g. and i.v. bolus dose of the agent. The glucuronide conjugates of THC and HHC have been identified previously in the bile of cannulated rats [Holder et al., 1978], but this is the first time that a metabolite containing the fully reduced diarylheptadienone chain, HHCOH, has been identified. Sulphate conjugates of the reduced metabolites, THC, HHC and HHCOH, were not detected in vivo, whereas THC and HHC glucuronide were detected in the plasma following i.v. dosing of rats. The conjugation of THC and HHC by glucuronidation and not sulphation may be the result of the greater relative abundance of the cofactors required for glucuronidation than of those required for sulphation (see section 1.4). HHCOH is the ultimate reduction product of curcumin and is potentially also a substrate for conjugating enzymes, however HHCOH
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sulphate and HHCYOH glucuronide were not identified during this study. Curcumin glucuronide, curcumin sulphate and curcumin glucuronide sulphate were also identified in the plasma following i.v. dosing of rats. In contrast, when curcumin was administered to rats as a single i.g. dose, the reduced metabolites, THC, HHC and HHCYOH, were below the limit of detection and curcumin sulphate, curcumin glucuronide and curcumin sulphate glucuronide were the major plasma metabolites. This is in agreement with the work of another in vivo study using Sprague-Dawley rats [Asai and Miyazawa, 2000], although the investigators did not report the occurrence of curcumin sulphate in the plasma.

Wang et al. investigated the stability of curcumin in vitro (see section 1.3.1) and found that the agent degraded to vanillin, ferulic acid and feruloyl methane at physiological pH. Rat plasma obtained following oral administration of curcumin (see section 5.2) was analysed for these degradation products using LC-MS as described in section 2.5. The degradation products were below the limit of detection.

In order to investigate whether administration of a single i.g. dose of curcumin was representative of dietary consumption of the agent, curcumin was administered to rats either by gavage or as a dietary constituent In the case of the gavage route of administration, curcumin was suspended in an amphiphilic solvent. The dose given to the rats by the two routes was calculated to be similar (see section 2.10.2). When curcumin was administered to rats as a single i.g. bolus, levels of curcumin in the plasma were approximately 3-5 times greater than those following dietary administration. In contrast, approximately 100 times more curcumin was measured in the colon mucosa following dietary feeding for 3 hr ("short feeding") than after i.g. intubation. The metabolites, curcumin sulphate and curcumin glucuronide, were only detected in the plasma following the i.g. route of administration. 

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These results show that administration of curcumin as a suspension is not reflective of that of curcumin admixed in the diet. This finding has two important implications. Firstly, most studies that have demonstrated the chemopreventive efficacy of curcumin have involved administration of the agent as a constituent of the diet, whereas investigations into its metabolism have been performed with the drug formulated in suspension using a variety of solvents such as 1% sodium cholate [Asai and Miyazawa, 2000], aqueous carboxymethyl cellulose solution [Pan et al., 1999] or arachis oils [Wahlstrom and Blenow, 1978] and administration via the i.g. or i.p. route. Secondly, it is clear that if curcumin is to be used to prevent tumours in tissues that are remote from the gastrointestinal tract, improvement of its oral bioavailability is necessary.

In an attempt to improve the bioavailability of curcumin, two formulations/prodrugs of curcumin were administered to rats and levels of curcuminoids were compared to those attained following administration of “sigma grade” curcumin. The first formulation, “Phytopharm P54” contained both essential oils and curcuminoids (see section 2.10.4). The second prodrug used was curcumin esterified with GLA. No improvement in the oral bioavailability of the agent was observed following administration of either of these formulations/prodrugs.

One of the major aims of this project was to determine whether sufficiently high levels of curcuminoids could be attained in the plasma of humans in order to elicit the pharmacological effects that have been demonstrated in vitro (see section 1.3.3). In order to test this hypothesis a dose-escalation study of “Phytopharm P54FP” in humans was carried out. Neither curcumin nor its metabolites were detectable in any of the blood fractions or urine of patients receiving a dose of up to 180 mg/day. The observed poor bioavailability of the agent
is consistent with studies in rats [Wahlstrom et al., 1978; Asai et al., 2000] mice [Pan et al., 1999] and in a pilot study in humans [Cheng et al., 2001]. Curcumin sulphate was identified in the faeces of one patient and this is the first time that a metabolite of curcumin has been detected \textit{in vivo} in humans.
6 General discussion
The results presented here allow several conclusions to be made with regard to the disposition and metabolism of the chemopreventive agent curcumin. These observations should assist the design and optimisation of future trials of curcumin. Firstly, the results from this study together with previously published work support the notion that the bioavailability of curcumin is poor, both in rats and humans. Following administration of an i.g. dose of 500 mg/kg to female F344 rats, the level of curcumin in the plasma was near the limit of detection. The low bioavailability of this agent has also been demonstrated recently following i.g. dosing of Sprague-Dawley rats [Asai and Miyazawa, 2000] and mice [Pan et al., 1999]. The results presented in sections 5.5 would suggest that the bioavailability of curcumin is also low in humans. In the pilot study of oral curcumin in humans described in section 5.5, curcumin could not be measured in the plasma of patients taking up to 180 mg of curcumin per day despite the limit of detection being as low as 0.1 ng/ml. Indeed in a pilot study in Taiwan, oral doses of 8,000 mg per day only generated plasma levels of 0.64 μg/ml [Cheng et al., 2001]. One consequence of the poor bioavailability of curcumin is accumulation of the agent in the gastrointestinal tract. Sufficiently high levels of curcumin to exert a pharmacological effect were attained in the colon mucosa and liver of rats following dietary administration of 2% curcumin (see section 5.3). Levels of M1G adducts were decreased in colon mucosa and levels of GST were increased in the liver [Sharma et al., 2001a]. The levels of curcumin measured in the colon are also sufficient to elicit the effects demonstrated in vitro (see section 1.3.3), including induction of apoptosis in the human colon cancer cells, HT-29 [Jiang et al., 1996], inhibition of COX-2 expression and NF-κB activity in the nonmalignant colon cell line, HCEC [Plummer et al., 1999]. Indeed the level of curcumin measured in the colon mucosa after 7 day feeding was 177 μg of curcumin per gram of tissue, which is approximately 100-fold higher than the minimum concentrations that have been shown to be
active in cells \textit{in vitro} [Plummer \textit{et al.}, 1999]. Work is currently underway at the Oncology Department, Leicester University, to investigate the safety and efficacy of higher doses of curcumin. Currently doses of up to 4 g of curcumin are being administered to patients diagnosed with colorectal or hepatic cancer without any curcumin-associated toxicity [Gescher \textit{et al.}, unpublished data]. After one week of ingestion of curcumin these patients undergo surgery, and curcumin levels are investigated in blood and surgical tissue. Thus far curcumin has been detected in the plasma but not in liver tissue or the urine of these patients [Gescher \textit{et al.}, unpublished data].

It is conceivable that the poor bioavailability of curcumin may at least in part be a result of its low aqueous solubility. If curcumin is to be advocated in the chemoprevention of tumours remote from the gastrointestinal tract, it is likely that formulation of the agent will be necessary. Microcrystallisation is a technique that can be used to improve the bioavailability of drugs. Micronised curcumin was administered to 18 volunteers formulated as a capsule or beverage (see section 5.5). At the dose levels used (50-200 mg per individual), curcumin was not detected in the plasma of patients. It is conceivable that other pharmaceutical formulations of curcumin may improve the bioavailability of the agent, although it may be an arduous task to attract commercial interest due to the impossibility of obtaining a patent for this dietary agent.

The second conclusion to be derived from this study is that the bioavailability and metabolism of curcumin are profoundly affected by the route of administration (see section 5.3). The level of curcumin in the plasma of rats was three fold higher when curcumin was administered by gavage than when an equivalent dose of curcumin was administered as a dietary constituent. In contrast, after dietary administration of curcumin, there was approximately
150-fold more curcumin in the colon than following an i.g. dose. Interestingly, the metabolites curcumin sulphate and curcumin glucuronide were only detected in the plasma following i.g. dosing. Although curcumin glucuronide has previously been identified as a plasma metabolite in rodents [Asai and Miyazawa, 2000; Pan et al., 1999] this is the first time that curcumin sulphate has been established as a curcumin metabolite in vivo (see Figure 6.4). Clearly administration of curcumin by gavage is not representative of dietary administration of the chemopreventive agent. This is an important observation because whilst pharmacokinetic studies have involved administration of curcumin as an i.g. bolus (see section 1.3.4), most chemopreventive studies described in section 1.3.2 have involved administration of the agent as a dietary constituent.

It is conceivable that the poor bioavailability of curcumin is at least in part a result of rapid metabolism. Indeed after administration of a single i.v. bolus to F344 rats, curcumin was found to be rapidly cleared from the plasma (see section 5.2). The liver has a major role in the metabolism of xenobiotic compounds. Wahlstrom et al. demonstrated that curcumin was rapidly metabolised by a suspension of isolated rat hepatocytes [Wahlstrom and Blenow, 1978], although the resulting metabolites were not identified. Suspensions of isolated rat and human hepatocytes reduced curcumin to HHC and HHCOH (see section 3.4). These metabolites were also generated in vivo following i.v. dosing of rats (see section 5.2). Whereas HHC has been identified previously in the bile of rats following i.v. dosing of rats [Holder et al., 1978], this is the first time that HHCOH has been described as a hepatic metabolite of curcumin (see section 3.4). The fact that curcumin has been demonstrated to be metabolised rapidly in vivo and in vitro, makes it imperative to understand whether the metabolites contribute to the biological efficacy of the agent. THC has been found to more potent that curcumin in the caragenin-induced rat paw oedema test for anti-inflammatory
activity [Mukhopadhay et al., 1982] and is at least as potent an antioxidant as curcumin in rabbit erythrocyte membrane ghosts and rat liver microsomes in vitro [Sugiyama et al., 1996; Osawa et al., 1995]. In contrast, THC was much less potent than curcumin as an inducer of quinone reductase in cells in vitro or as an inhibitor of TPA-induced tumour promotion in mouse skin [Huang et al., 1995]. The metabolites THC, HHC and HHCOH were also found to be less potent than curcumin in reducing levels of iNOS in LPS stimulated macrophages [Pan et al., 2000]. Interestingly, when curcumin was used to protect against paracetamol induced hepatocyte toxicity, the same effect was observed whether the hepatocytes were treated with curcumin 1 hr before, concomitantly, or 1 hr after addition of paracetamol to the cells [Donatus et al., 1990]. In the situation where curcumin was added to the cells 1 hr prior to addition of paracetamol, it is likely that the majority of the compound will have been metabolised to HHC and HHCOH (see section 3.4.1). Therefore one possible indirect conclusion would be that curcumin and its reduced metabolites, HHC and HHCOH, have similar efficacy in preventing paracetamol toxicity. The ability of curcumin to inhibit the expression of inducible COX-2 is another potential mechanism of this agent’s cancer chemopreventive activity [Plummer et al., 1999]. To determine whether the metabolites identified share this pharmacological property with curcumin, COX-2 expression was induced by PMA in the normal colon cell line HCEC which had been pre-incubated with curcumin or its metabolites, THC, HHC, HHCOH or curcumin sulphate [Ireson et al., 2001]. Curcumin was found to be more potent than its metabolites in its ability to inhibit inducible COX-2 expression. The order of potency of inhibition of COX-2 expression of the curcuminoids was: curcumin>HHC>THC>curcumin sulphate and HHCOH [Ireson et al., 2001]. Clearly, the plasma and hepatocytic metabolites of curcumin are less capable than the parent compound in terms of inhibition of COX-2 expression [Ireson et al., 2001] and consequently Figure 6.4 could be considered as deactivation pathway.
The results described in Chapter 3 clearly demonstrate that the gastrointestinal tract plays a major role in the biotransformation of curcumin. This is an agreement with the early observations of Ravindranath et al. who observed metabolism of $[^3\text{H}]$-radiolabelled curcumin by everted rat gut sacs [Ravindranath and Chandrasekhara, 1981], but did not identify the metabolites generated. This is the first time that metabolism of curcumin by glucuronidation, sulphation and reduction by rat and human intestinal tissue has been unequivocally demonstrated. It is therefore possible to postulate that the poor bioavailability of curcumin may partly be a consequence of rapid extrahepatic metabolism. Indeed Shoba et al. demonstrated that higher levels of curcumin could be attained in the plasma of humans and rats if the agent was co-administered with piperine, an inhibitor of intestinal and hepatic glucuronidation [Shoba et al., 1998]. Recently, the bioavailability of a $\beta$-ketone analogue of curcumin, dibenzoylmethane (see Figure 6.2), has been shown to be greater than that of curcumin [Lin et al., 2001]. The authors concluded that the improved bioavailability may be due to the absence of unsaturated alkyl and phenolic groups in the molecule, which are potential sites of reduction and conjugation respectively. To establish whether the presence of a GLA group attached to each of the phenolic groups (section 2.10.4) might hinder intestinal metabolism and result in increased bioavailability of the agent, curcumin GLA ester was administered to rodents by gavage and levels of curcumin in the plasma were compared to those measured following administration of a curcumin suspension. There was no increase in the levels of curcumin measured in the plasma following administration of curcumin GLA ester.

Following administration of a single oral dose (500 mg/kg) of curcumin to F344 rats, only low levels of parent compound could be detected in the plasma (see section 5.2), which
suggests poor oral bioavailability of this agent. Curcumin is a lipophilic compound and consequently it should be able to cross the plasma membrane, enter the intestinal cells and reach the hepatic portal vein (see Figure 6.1). Indeed Plummer et al. added curcumin to the human colonic cell lines, HCA-7 and HCEC and determined that approximately 50% of the agent was taken up by the cells [Plummer et al., unpublished observation]. It is conceivable therefore that the low levels of the agent in the plasma result from processes that occur during the passage of curcumin from the gut lumen to the blood. Recent studies have demonstrated that isoforms of the CYP3A subfamily are expressed in human small intestine [Thummel et al., 1997]. CYP3A (see Figure 6.1), expressed in the small intestine, has been shown to metabolise felodipine and reduce its oral bioavailability [Regardh et al., 1989]. However incubations of curcumin with intestinal microsomes fortified with the cofactors necessary for cytochrome P450 metabolism did not yield detectable levels of metabolites (result not shown). The multiple drug resistance 1 (MDR1) P-glycoprotein (P-gp) (see Figure 6.1) has also been shown to be expressed in intestinal enterocytes [Ambudkar et al., 1999]. This transmembrane protein is thought to reduce the oral bioavailability of drugs by transporting the agents from enterocytes into the intestinal lumen. Knock out mice have been used to demonstrate the role of this glycoprotein in reducing the oral bioavailability of drugs such as the HIV protease inhibitors indinavir and nelfinavir [Kim et al., 1998] and paclitaxel [Sparrenboom et al., 1997]. In these studies the agents were administered orally to mdr1 (-/-) knockout mice and normal mice and the area under the curve (AUC) values were compared. The normal mice had significantly lower oral bioavailability than the mdr1 knockout mice. The mulitdrug resistance associated protein (MRP) family of transporters (see Figure 6.1) have been shown to remove organic anions including conjugated metabolites (e.g. 1-chloro-2,4-dinitrobenzene) from enterocytes [Suzuki and Sugiyama, 1998]. Curcumin has been shown to be metabolised to curcumin glucuronide and curcumin sulphate by human and rat
intestinal subcellular fractions (see section 3.2) and intact rat gut (see section 3.3). It would be interesting to experimentally determine whether curcumin conjugates are substrates for the MRP family of transporters and also whether curcumin is a substrate for P-gp. It is possible that these transporters may contribute to the poor oral bioavailability of curcumin although this hypothesis remains to be experimentally confirmed.
Figure 6.1. Schematic representation illustrating the synergistic role of metabolic enzymes and efflux transporters in intestinal cells. (A) Synergistic role of CYP 3A and MDR1 P-gp. Following uptake into the cell, drugs can be metabolised by CYP3A isoenzymes or eliminated from the cell by P-gp. (B) Synergistic roles of conjugative enzymes (e.g. SULT and UGT) and efflux transporters for organic anions. After being taken up into the cell, drug molecules may be metabolised by to generate glucuronide and sulphate conjugates. These metabolites may be eliminated from the cell by transporters for organic anions, e.g. MRP 2 and 3 and possibly other transporters that are yet to be identified. Figure adapted from Suzuki et al., 2000.
Another important conclusion of this work was that significantly more HHC, curcumin sulphate and curcumin glucuronide were generated by human intestinal subcellular fractions than from the equivalent rat tissue. Therefore it seems possible to suggest that levels of unmetabolised curcumin attained in the plasma \textit{in vivo} in rodents at a particular dose of the agent may be an overestimation of the values that would be attained in humans. Curcumin sulphate is the first curcumin metabolite to be identified in humans \textit{in vivo} (see section 5.5). This metabolite was only detected in faeces and not in any of the blood constituents and therefore it is possible that this species may have been generated extrahepatically in the gastrointestinal tract.

Figure 6.2. Chemical structure of dibenzoylmethane
The rapid metabolism of curcumin also has implications for other polyphenolic chemopreventive agents which have comparable chemical structural features, for example genistein and resveratrol (see Figure 6.3). Indeed genistein sulphate [Coldham et al., 1999] and genistein glucuronide [Coldham et al., 1999; Sfakianos et al., 1997] and resveratrol glucuronide [Kuhnle et al., 2000] have been identified in vivo. It is conceivable that other polyphenolic chemopreventive agents undergo similar metabolic routes.
Figure 6.3. Chemical structures of the chemopreventive agents resveratrol and genistein
Chapter 6

Several novel metabolites have been identified during this study (see Figure 6.4), but little is understood with regard to the enzymes involved in the generation of these metabolites. The results presented in Chapter 4 give some insight into the enzymes in the metabolic generation of curcumin sulphate and HHC. In order to gain an understanding of the enzymatic generation of curcumin sulphate in humans, two human recombinant SULT isoforms, SULT1A1 and 1A3, were incubated with curcumin. Both isoforms were found to conjugate curcumin to generate curcumin sulphate and were shown to be expressed in human gut. Alcohol dehydrogenase was also shown to have a role in the metabolic biotransformation of curcumin to HHC, although it is likely that other non-specific reductases may play a role in the reduction of curcumin \textit{in vivo}. 
Figure 6.4. Overview of the metabolic pathway of curcumin *in vivo* in rats. The use of red indicates that these were novel metabolites identified in rat plasma during this study. The use of italics indicates that these metabolites were also identified *in vitro*.
In conclusion, the results from this study demonstrate that curcumin is not bioavailable in humans or rodents. The lack of bioavailability is probably a consequence of its rapid extrahepatic and hepatic metabolism and its poor aqueous solubility. However, sufficiently high levels of unmetabolised curcumin are likely to be achieved in the gastrointestinal tract to be useful in terms of human colorectal cancer chemoprevention. If oral curcumin is to be advocated in the chemoprevention of tumours remote from the gut then careful consideration will have to be given to preparation of a formulation that will improve its oral bioavailability.
Appendix-Publications
Publications

Full papers


Abstracts


Publications


Metabolism of the Cancer Chemopreventive Agent Curcumin in Human and Rat Intestine

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Abstract

Curcumin, the yellow pigment in turmeric, prevents malignancies in the intestinal tract of rodents. It is under clinical evaluation as a potential colon cancer chemopreventive agent. The systemic bioavailability of curcumin is low, perhaps attributable, at least in part, to metabolism. Indirect evidence suggests that curcumin is metabolized in the intestinal tract. To investigate this notion further, we explored curcumin metabolism in subcellular fractions of human and rat intestinal tissue, compared it with metabolism in the corresponding hepatic fractions, and studied curcumin metabolism in situ in intact rat intestinal sacs. Analysis by high-performance liquid chromatography, with detection at 420 or 280 nm, permitted characterization of curcumin conjugates and reduction products. Chromatographic inferences were corroborated by mass spectrometry. Curcumin glucuronide was identified in intestinal and hepatic microsomes, and curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin were found as curcumin metabolites in intestinal and hepatic cytosol from humans and rats. The extent of curcumin conjugation was much greater in intestinal fractions from humans than in those from rats, whereas curcumin conjugation was less extensive in hepatic fractions from humans than in those from rats. The curcumin-reducing ability of cytosol from human intestinal and liver tissue exceeded that observed with the corresponding rat tissue by factors of 18 and 5, respectively. Curcumin sulfate was identified in incubations of curcumin with intact rat gut sacs. Curcumin was sulfated by human phenol sulfurtransferase isoenzymes SULT1A1 and SULT1A3.

Equine alcohol dehydrogenase catalyzed the reduction of curcumin to hexahydrocurcumin. The results show that curcumin undergoes extensive metabolic conjugation and reduction in the gastrointestinal tract and that there is more metabolism in human than in rat intestinal tissue. The pharmacological implications of the intestinal metabolism of curcumin should be taken into account in the design of future chemoprevention trials of this dietary constituent.

Introduction

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the herb Curcuma longa Linn. In the Indian subcontinent and Southeast Asia, turmeric has traditionally been used as a treatment for inflammation, skin wounds, and tumors. Clinical activity of curcumin has yet to be confirmed; however, in preclinical animal models, curcumin has shown cancer chemopreventive, antiinflammatory, and anti-inflammatory properties (for review, see Ref. 1). Especially interesting is its ability to prevent the formation of carcinogen-induced intestinal premalignant lesions and malignancies in rats (2, 3) and in the multiple intestinal neoplasia (Min+) mouse (4), a genetic model of the human disease familial adenomatous polyposis. Curcumin acts as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen (5–9), and it interferes with lipid peroxidation (10–12). Curcumin suppresses a number of key elements in cellular signal transduction pathways pertinent to growth, differentiation, and malignant transformation. Among signaling events inhibited by curcumin are protein kinases (13), c-Jun/AP-1 activation (14), prostaglandin biosynthesis (15), and activity and expression of the enzyme cyclooxygenase-2 (16). This latter property is probably mediated via the ability of curcumin to block activation of the transcription factor NF-κB at the level of the NF-κB-inducing kinase/IKKα/β signaling complex (17). In rodents, curcumin demonstrates poor systemic bioavailability after p.o. dosing (18), which may be related to its inadequate absorption and avoid metabolism. Curcumin bioavailability may also be poor in humans, as borne out by a recent pilot study of a standardized Curcuma extract in colorectal cancer patients (19). After p.o. dosing, curcumin undergoes metabolic O-conjugation to curcumin glucuronide and curcumin sulfate and bioreduction to tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol (Fig. 1) in rats and mice in vivo (18, 20, 21) and in suspensions of human and rat hepatocytes (18). Products of curcumin reduction are also subject to glucuronidation (20). Certain curcumin metabolites, such as tetrahydrocurcumin, possess anti-inflammatory (11) and antioxidant activities (22, 23) similar to those of their metabolic progenitor. It has been suggested that the intestinal tract plays an important role in the metabolic disposition of curcumin. The evidence supporting this notion is based predominantly on experiments in which [3H]labeled curcumin was incubated with inverted rat gut sacs, and

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Biotransformation was deduced from the disappearance of radioactivity associated with the parent compound (24). Metabolites of curcumin have hitherto not been unambiguously identified in gut tissue. Therefore, we tested the hypothesis that curcumin is metabolically conjugated and/or reduced in intestinal tissue. This hypothesis was tested using two experimental settings: cytosolic and microsomal fractions of intestinal tissue obtained from humans and rats and suspensions of intact rat gut sacs under the conditions used to make the original observations regarding the intestinal metabolism of $^{3}$H-curcumin (24). To be able to obtain an indication of the quantitative importance of the contribution of intestinal tissue to the overall metabolism of curcumin in the organism, the extent of its biotransformation in intestinal tissue was compared with that in analogous liver fractions. Because the knowledge of enzymatic details of curcumin biotransformation is only rudimentary, we also addressed the question as to which enzymes may be involved in the metabolic generation of curcumin sulfate and hexahydrocurcumin, two major curcumin metabolites that we identified in intestinal tissue. Overall, the experiments were designed to contribute to the body of knowledge that will ultimately help rationalize the design of future chemoprevention trials of curcumin.

Materials and Methods

Chemicals and Reagents. The following chemicals and reagents were purchased from the suppliers listed: curcumin, uridine 5'-diphosphoglucuronic acid, magnesium chloride, PAPS, adenosine 3',5'-diphosphate, NADPH, equine alcohol dehydrogenase, and Triton X-100: Sigma Chemical Co.-Aldrich Comp., Ltd. (Poole, Dorset, United Kingdom). Authentic curcumin glucuronide, curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin were synthesized as described (18), and the latter two were provided by Dr. W. Wang (Phytopharm plc, Godmanchester, United Kingdom). In experiments in which the metabolism of curcumin or hexahydrocurcumin was studied in incubations with tissue fractions, gut sacs, or enzymes, substrates were dissolved in DMSO, and an aliquot of 5 μl was added to incubate to furnish a final substrate concentration of 100 μM.

Purification of PAPS. The purity of the purchased PAPS was determined by HPLC to be only 80%. Commercial PAPS is often contaminated with phosphoadenosine 5'-phosphate, which inhibits SULT enzymes (25). To remove this contaminant from PAPS, the commercial product was purified by HPLC, essentially as described previously (26) using a Varian Prostar (310 model) solvent delivery system coupled to an octadecyl silan reversed phase C18 column (4.6 × 250 mm; Beckman) and a UV-visible detector. Aliquots (100 μl) of the PAPS solution (4 mM) were injected into the column. The eluant flow rate was 1.3 ml/min. Eluant was collected on dry ice, and PAPS was concentrated by rotary evaporation (4 min, room temperature). The collected PAPS was >99% pure by HPLC analysis.

Preparation of Rat and Human Intestinal and Hepatic Microsomes and Cytosol. Experiments using animals were conducted as stipulated by Project License 80/1250 granted to the MRC Toxicology Unit by the United Kingdom Home Office, and the experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation. Male F344 rats were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture. Liver and intestine were removed and snap frozen in liquid nitrogen. Human tissue was obtained from the United Kingdom Human Tissue Bank (Leicester, United Kingdom); healthy tissues had been resected from 6 Caucasian patients (livers from 1 male, who was 4 years of age, and 2 females, 30 and 51 years of age; intestine from 3 females, who were 29, 54, and 56 years of age). Patients had not received medication known to interfere with xenobiotic metabolism activity. Human and rat intestinal or hepatic tissue was defrosted and weighed, and microsomes and cytosol were prepared as described previously (27). Human intestinal tissue originated from the jejunal area of the intestine, and rat intestinal tissue came from the jejunum and colon.

Metabolism of Curcumin by Intestinal and Hepatic Subcellular Fractions. To study curcumin conjugation, substrate (100 μM) was incubated for 1 h with hepatic or intestinal cytosol or microsomes (1 mg of cytosolic or microsomal protein/ml) in phosphate buffer (0.01 M) at 37°C. Cytosolic or microsomal protein was quantitated using the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany). Incubations to study glucuronidation included microsomes, uridine 5'-diphosphoglucuronic acid (3 mM), magnesium chloride (5 mM), and Triton X-100 (0.01%) in phosphate buffer at pH 7.4. Incubations to study sulfation included cytosol with PAPS (0.4 mM) and mercaptoethanol (5 mM) in phosphate buffer at pH 8.4. Curcumin sulfate and curcumin glucuronide were quantitated with the help of a calibration curve established using curcumin. In orientation experiments, curcumin glucuronide and sulfate generation were found to be linear for ≤30 min, after which the rate of conjugate formation declined. Therefore, the values shown in Table 1 are amounts generated per h. To study curcumin bioreduction, substrate (100 μM) was incubated with cytosol or microsomes and NADPH (1 mM) in phosphate buffer (10 mM, pH 7.4) in a final volume of 0.5 ml at 37°C. Incubation time was 90 min, during which generation of hexahydrocurcumin was linear. The amount of hexahydrocurcumin generated was assessed using a calibration curve with authentic hexahydrocurcumin. In control experiments, substrate and reaction components were incubated with microsomal or cytosolic fractions in which enzymes had been inactivated by exposure to boiling water for 10 min. Reactions were terminated by cooling incubate samples to −80°C.

Metabolism of Curcumin by Intact Rat Intestine. The jejunal section of the small intestine of terminally anesthetized male F344 rats (180 grams) was excised. Gut content was
removed by flushing with 0.9% (w/v) sodium chloride solution. Everted gut sacs of ~8-cm length were prepared as described previously (28) using a glass rod. Sacs were suspended in an Ussing chamber in Krebs-Ringer phosphate buffer (10 ml), containing glucose (10 mM) and curcumin to give a final concentration of 100 μM. Buffer with substrate was added to the lumen of the gut to ensure full extension. Gut sacs were incubated with curcumin for 1 h under a continual stream of carbogen at 37°C.

**Metabolism of Curcumin by Isolated Enzymes and Western Analysis.** Curcumin (100 μM) was incubated with recombinant SULT1A1 and 1A3 (10 μg/ml) obtained as described previously (29) or with equine alcohol dehydrogenase (10 units/ml) in phosphate buffer (0.01 mM, pH 4.4 for sulfation, pH 7.4 for reduction); the final volume was 0.5 ml. Incubations contained PAPS (0.4 mM, sulfation) or NADPH (10 mM, reduction) and were conducted at 37°C for 1 h. Metabolites were extracted as described above. SULT1A1/1A3 primary antibodies were prepared and used for Western blotting as described previously (29).

**HPLC Analysis.** After acidification with acetic buffer (1 M, pH 4.6), samples were extracted twice with ethyl acetate:propan-2-ol (9:1), and mixtures were centrifuged (2800 × g, 4°C, 15 min). 3,10,15,20-Tetra-(m-hydroxyphenyl)-chlorine served as internal standard. The organic layers were removed and combined, and solvent was evaporated to dryness under nitrogen. A reversed-phase HPLC method described before (18) was used to determine the quantity of curcumin and its metabolites. HPLC analysis was performed on a Vartan Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410) and a Symmetry Shield RP 18 column (150 × 3.9 mm, Waters). Detection of curcumin, curcumin sulfate, and curcumin glucuronide was achieved at 420 nm, whereas tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcumol were analyzed in negative ion mode. The extraction efficiency for hexahydrocurcumin from incubations with tissue fractions was 92 ± 7% (mean ± SD, n = 6), which for curcumin was very similar (18). In contrast, the extraction efficiencies for curcumin glucuronide and curcumin sulfate were only 50 ± 9%.

**Mass Spectrometry.** Mass spectrometry was performed as described before (18) using a Quattro Bio-Q tandem quadrupole mass spectrometer upgraded to Quattro MX II specifications (Micromass, Altrincham, Cheshire, United Kingdom) with a pneumatically assisted electrospray interface. Samples were analyzed in negative ion mode. The temperature was maintained at 120°C, the operating voltage of the electrospray capillary was 3.88 kV, and the cone voltage was 32 V. HPLC conditions used for the on-line HPLC-mass spectrometric analyses were as described before (18).

**Results**

**Curcumin Metabolism in Subcellular Fractions of Intestine and Liver.** Curcumin was incubated with intestinal and hepatic cytosol and microsomes obtained from humans and rats. Extracts of the incubation mixtures were analyzed by HPLC for curcumin, and its metabolites were analyzed with detection by UV/visible spectrophotometry at 420 and 280 nm and by mass spectrometry in the selected ion monitoring mode. Spectrophotometric analysis at 420 nm indicated the presence of molecules, such as curcumin sulfate and curcumin glucuronide, containing the intact yellow-colored diferuloylmethane structure, whereas detection at 280 nm also allowed characterization of molecules, such as tetrahydrocurcumin and hexahydrocurcumin, generated from curcumin by reduction destruction of the chromophoric diarylheptatrienone chain. Metabolite analysis was aided by chromatographic comparison with authentic reference compounds. Analysis of incubates of curcumin with human and rat intestinal cytosol (Fig. 2) yielded curcumin sulfate and hexahydrocurcumin. Both species were characterized mass spectrometrically by molecular ions of m/z = 447 and 373, respectively. Fig. 3A shows the selected ion chromatogram of curcumin sulfate generated in human gut cytosol. There was also evidence of the presence of tetrahydrocurcumin as adjudged by mass spectrometry (molecular ion m/z = 371). On UV-spectrophotometric detection, authentic tetrahydrocurcumin gave a broad shoulder with a nonsymmetrical peak, probably the corollary of an unstable equilibrium of stereoisomers, which are possible for the 1,7-diarylhepta-(3,4-ene)-5-one structure (Fig. 1). Furthermore, a small peak in the cytosolic extracts eluted at the retention time of authentic hexahydrocurcumolin (retention time: 22 min), but this species eluded conclusive identification. HPLC analysis of extracts of incubates of curcumin with human or rat intestinal microsomes (Fig. 2) afforded a peak consistent with curcumin glucuronide, as adjudged by its chromatographic properties and its molecular ion of m/z = 543. Microsomes did not generate detectable levels of products of curcumin reduction. Results qualitatively similar to those shown in Fig. 2 were obtained with hepatic cytosol and microsomes (results not shown).

**Quantitation of Curcumin Metabolites.** Quantitative analysis revealed considerable differences in curcumin metabolite generation between human and rat tissue and between gut and liver when values were normalized to cytosolic or microsomal protein content (Table 1). The extent of sulfation of curcumin in the cytosol of human intestinal tissue was four times that in rat intestine, whereas in human liver cytosol, it was only a fifth of that observed in rat liver cytosol. Curcumin sulfation was 3-fold higher in cytosol from human intestine than in that from human liver, whereas in the rat, intestinal sulfation was only a seventh of that in the liver. Microsomal metabolism of curcu-
min generated as much as 16 times more curcumin glucuronide in the intestine of humans than in the equivalent tissue in the rat, but human liver microsomes generated only a third of the amount of curcumin glucuronide found in rat liver microsomes. Micromosal glucuronidation of curcumin in human intestine exceeded that seen in liver by a factor of 2.5, whereas in the rat, the amount of curcumin glucuronide formed in the intestine was only a 60th of that measured in the liver. There was considerable variation in formation of curcumin glucuronide in human intestine, perhaps related to interindividual differences in the abundance of UDP-glucuronosyl-transferase enzymes influenced by the donor's genotype, disease state, and lifestyle. Reduction of curcumin to hexahydrocurcumin was 18 times higher in human intestinal cytosol and five times more abundant in human liver cytosol compared with the corresponding values in rats. Production of hexahydrocurcumin occurred to a similar extent in the cytosol of human intestinal and hepatic tissue. In the rat, reduction of hexahydrocurcumin in the intestine was only a third of that seen in liver.

To study topological differences between rat gut segments in ability to metabolize curcumin, extent of metabolism in cytosol and microsomes was compared between rat jejunum and colon. The amounts of curcumin sulfate generated by jejunal or colonic cytosol were 25 ± 3 and 40 ± 9 nmol/mg protein (mean ± SD, n = 3), respectively. Reductive metabolism in cytosol from the jejunum furnished 8 ± 3 nmol hexahydrocurcumin/mg protein, whereas the amount of hexahydrocurcumin detected in colonic cytosol was below the limit of quantification. Microsomes from the jejunum or colon generated 16 ± 1 and 42 ± 11 nmol curcumin glucuronide/mg protein, respectively (mean ± SD, n = 3).

The enzymes SULT1A1 and 1A3 are among five isoenzymes of the human phenol xenobiotic-metabolizing SULT subfamily, which are expressed in the gastrointestinal tract (30). To explore whether they may be involved with the generation of curcumin sulfate in tissue fractions, curcumin was incubated with recombinant SULTs. Analysis of extracts of the incubates by HPLC-mass spectrometry confirmed that SULT1A1 (Fig. 3) and SULT1A3 (result not shown) metabolize curcumin to its

**Fig. 3.** High-performance liquid chromatograms of extracts of incubations of curcumin (100 µM) with cytosol (A and C) and microsomes (B) from human intestinal tissue and with cytosol (D and F) and microsomes (E) from rat intestinal tissue. Incubation periods were 90 min for metabolic reduction (A and D) and 60 min for conjugation (B, C, E, and F). Chromatographic analysis was conducted at 280 (A and D) and 430 nm (B, C, E, and F). The identity of the peaks was established by cochromatography and mass spectrometry as curcumin (1), hexahydrocurcumin (2), curcumin glucuronide (4), and curcumin sulfate (5). The prominent peak labeled "is" (retention time: 51 min) was caused by the internal standard 5,10,15,20-tetra-(m-hydroxyphenyl)-chlorine. Note that commercially available curcumin contains 13% dimercurcurcumin and 3% bisdermethoxycurcumin, which furnished two small peaks just beyond curcumin (see especially in A and D). Removal of the methoxy moieties from the curcumin molecule appears to render its phenolic groups more resistant against metabolic conjugation, and bisdesmethoxycurcumin was not a substrate of the conjugating enzymes. AU, absorbance units. For details of incubation, extraction, and HPLC analysis, see “Materials and Methods.” The chromatograms are representative of analyses conducted with fractions or tissues from three humans or four rats.

*Fig. 2.* On-line HPLC-mass spectrometry analysis in selected ion registration mode of curcumin sulfate (molecular ion at m/z 647) derived from extracts of incubations of curcumin with human intestinal cytosol (A) and recombinant SULT1A1 (B). Arrow, retention time of authentic curcumin sulfate. For details of incubation, extraction, and HPLC analysis, see "Materials and Methods." The chromatograms are representative of analyses conducted with fractions or tissues from three humans or four rats.
sulfate. At a curcumin substrate concentration of 100 μM, 6 nmol curcumin sulfate/μg protein were generated by SULT1A1 during the 30-min incubation (mean of n = 2), and SULT1A3 catalyzed the production of four times this amount of curcumin sulfate. Western blot analysis corroborated the presence of both SULT1A1 and 1A3 in human intestinal and hepatic cytosol (Fig. 4).

To find out if alcohol dehydrogenases may be involved in curcumin reduction, curcumin was incubated with horse alcohol dehydrogenase. Hexahydrocurcumin was identified as a metabolite by HPLC and mass spectrometry (result not shown). Both hexahydrocurcumin and hexahydrocycrocinol have been reported to be the predominant products of metabolic reduction of curcumin in incubations of intact human or rat hepatocytes (18), whereas in the experiments with liver or gut curcumin described above, hexahydrocurcumin and tetrahydrocurcumin were the major curcumin reduction products, and only traces of hexahydrocycrocinol were found. To rationalize this difference in metabolism of curcumin between cellular cytosol on the one side and intact cells on the other, curcumin or hexahydrocurcumin was incubated with human gut microsomes, fortified with NADPH-generating cofactors. Fig. 5 shows that microsomes reduced hexahydrocurcumin to hexahydrocycrocinol but did not metabolize curcumin. This result means that whereas curcumin is a poor substrate of human microsomal reducing enzymes, hexahydrocurcumin is a good substrate of these enzymes. In contrast, human gut cytosol reduced curcumin easily (see Fig. 2, and it also reduced hexahydrocurcumin to hexahydrocycrocinol (result not shown).

Discussion
These are the first results to provide convincing evidence that curcumin is biotransformed in the intestinal tract of humans and rodents. Metabolism of curcumin to curcumin glucuronide, curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin was demonstrated in intestinal fractions from humans and rats, and its conversion to curcumin sulfate was demonstrated in situ in intact rat intestine. These findings confirm and extend the original observations that in rat gut sacs, in situ [3H]labeled curcumin underwent metabolic removal from the incubation medium (23). They also corroborate the finding that intestinal mucosa, as well as liver and kidney tissue from the rat, can glucuronidate and sulfate curcumin, as adjudged by analysis of differential amounts of curcumin present before and after treatment of tissue extracts with conjugate-hydrolyzing enzymes (21). The results of the quantitative evaluation of curcumin metabolism in human and rat tissue fractions presented in Table 1 suggest that gut metabolism contributes substantially to the overall metabolite yield generated from curcumin in vivo. Furthermore, they support the notion that the colon may be more capable of conjugating curcumin than the jejunum, at least in the rat. The data shown in Table 1 allow a comparison to be made between humans and rats as to the capability of intestinal and hepatic tissues to metabolize curcumin, thus helping define the suitability of the rat as a model to study the metabolism of curcumin in humans. Whereas the pattern of metabolites of curcumin in human intestinal and hepatic tissues was qualitatively similar to that in rat tissues, there were considerable quantitative differences. In human intestinal fractions, conjugation of curcumin with activated sulfite or glucuronic acids was much more abundant, whereas conjugation in human hepatic fractions was much less extensive, than in rat tissues. Furthermore, the ability of either intestinal or liver tissues from humans to reduce curcumin exceeded that in tissues from rat by factors of 18 and 5, respectively. These differences may reflect discrepancies in tissue enzyme content. Taken together, these results suggest that experiments in the rat may severely underestimate the extent of intestinal metabolism of curcumin, which occurs in humans, a conclusion which hints at the possibility that, in quantitative terms, the rat may not be a good model for the elucidation of the extrahepatic metabolic disposition of curcumin in humans.

The results also provide preliminary insights into the enzymology associated with intestinal metabolism of curcumin. Curcumin was shown to be a substrate of both phenol SULTs tested here, SULT1A1 and SULT1A3. Of the two, the latter was more efficient in sulfating curcumin. Curcumin sulfate is the first curcumin metabolite that has been identified in human feces (19), and its generation may have been catalyzed by these enzymes in the gut. Alcohol dehydrogenase was pinpointed here as a potential source of metabolically generated hexahydrocurcumin. Nevertheless, it is probable that a variety of other ubiquitous and nonspecific oxidoreductases reduce curcumin and thus contribute to the formation of curcumin reduction products in vivo. The potential involvement of alcohol dehydrogenase enzymes with the cytosolic reduction of curcumin in gut and liver potentially raises the clinically pertinent question of whether the metabolic disposition of curcumin may be compromised by consumption of alcoholic beverages and, if
In the testinal Metabolism of Curcumin in intestinal and hepatic cytosol (open arrows) and microsomes (closed arrows).

Fig. 6. Schematic representation of metabolism of curcumin in intestinal and hepatic cytosol (open arrows) and microsomes (closed arrows).

versa, that dehydrogenation of dietary alcohol may be impaired in individuals who consume large amounts of curcumin.

The cytosolic and microsomal components of the metabolic disposition of curcumin and their interrelationship as demonstrated by the results described above are highlighted in Fig. 6. The enzymatic source of curcumin reduction products was found to be cytosolic exclusively, whereas enzymatic reduction of the curcumin metabolite hexahydrocurcumin to hexahydrocurcuminol was catalyzed by both cytosolic and microsomal enzymes. The differential subcellular compartmentalization with respect to curcumin reduction may help explain why its complete reduction to hexahydrocurcuminol, which involves four two-electron reduction steps and proceeds probably via dihydrocurcumin, tetrahydrocurcumin, and hexahydrocurcumin as intermediates, seems to occur more efficiently in the intact cell (18) than is reflected by the experiments described here using isolated subcellular fractions. A candidate enzyme responsible for the final reduction step, the generation of hexahydrocurcuminol from hexahydrocurcumin, in microsomes is cytochrome P450 reductase, but this suggestion needs to be experimentally confirmed.

The results presented above have a number of potentially important pharmacological implications, which may impinge on the design of future clinical trials of curcumin. The prime implication pertinent to curcumin pharmacokinetics is that avid intestinal sulfation, glucuronidation, and reduction, especially in humans, may well be a major reason for its poor systemic availability. This situation is probably analogous to the low bioavailability of drugs, such as the oral contraceptive ethinylestradiol, which is thought to be caused by extensive sulfate conjugation (31). It needs to be noted though that the poor bioavailability of curcumin is probably to a great extent the consequence of its deficient pharmacutical profile, exemplified by extremely low aqueous solubility.

Tetrahydrocurcumin was found to be more potent than curcumin in the carrageenin-induced rat paw edema test for anti-inflammatory activity (11) and at least as potent an antioxidant in rabbit erythrocyte membrane ghosts and rat liver microsomes in vitro (22, 23). In contrast, tetrahydrocurcumin was much less potent than curcumin as an inducer of quinone reductase in cells in vitro (32) or as an inhibitor of phorbol 12-myristate 13-acetate-induced tumor promotion in mouse skin (33). Furthermore, in a comparative analysis of the ability of five curcumin metabolites, curcumin sulfate, curcumin glucuronide, tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol, to interfere with phorbol ester-induced expression of the enzyme cyclooxygenase-2, none of these species was found to be as potent as their metabolic progenitor (18). On balance, these findings justify the tentative conclusion that the intestinal biotransformation of curcumin constitutes a pharmacological deactivation process, in that metabolites creates species that are either devoid of biological activities germane to cancer chemoprevention or less potent than their metabolic precursor.

In conclusion, this study demonstrates that curcumin is avidly metabolized by human intestinal tissue. The pharmacological implications of intestinal conjugation and bioreduction of curcumin should be considered in the design of future cancer chemoprevention trials of this interesting dietary constituent.

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Pharmacodynamic and Pharmacokinetic Study of Oral *Curcuma* Extract in Patients with Colorectal Cancer

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**ABSTRACT**

*Curcuma* spp. extracts, particularly the dietary polyphenol curcumin, prevent colon cancer in rodents. In view of the sparse information on the pharmacodynamics and pharmacokinetics of curcumin in humans, a dose-escalation pilot study of a novel standardized *Curcuma* extract in proprietary capsule form was performed at doses between 440 and 2200 mg/day, containing 36–180 mg of curcumin. Fifteen patients with advanced colorectal cancer refractory to standard chemotherapies received *Curcuma* extract daily for up to 4 months. Activity of glutathione S-transferase and levels of a DNA adduct (M1 G) formed by malondialdehyde, a product of lipid peroxidation and prostaglandin biosynthesis, were measured in patients' blood cells. Oral *Curcuma* extract was well tolerated, and dose-limiting toxicity was not observed. Neither curcumin nor its metabolites were detected in blood or urine, but curcumin was recovered from feces. Curcumin sulfate was identified in the feces of one patient. Ingestion of 440 mg of *Curcuma* extract for 29 days was accompanied by a 59% decrease in lymphocytic glutathione S-transferase activity. At higher dose levels, this effect was not observed. Lenkocytic M1G levels were constant within each patient and unaffected by treatment. Radiologically stable disease was demonstrated in five patients for 2–4 months of treatment. The results suggest that (a) *Curcuma* extract can be administered safely to patients at doses of up to 2.2 g daily, equivalent to 180 mg of curcumin; (b) curcumin has low oral bioavailability in humans and may undergo intestinal metabolism; and (c) larger clinical trials of *Curcuma* extract are merited.

**INTRODUCTION**

Curcumin (diferuloylmethane), a low molecular weight polyphenol derived from the rhizomes of *Curcuma* spp., has been shown to prevent cancer in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (1). Of particular interest is the ability of dietary curcumin to interfere with colon carcinogenesis in chemical and genetic rodent models (2–4). Curcumin has also been associated with regression of established malignancy in humans (5). Curcumin is the major constituent of the spice turmeric, which is abundantly used in the diet on the Indian subcontinent, an area that has a low incidence of colorectal cancer (6). Mechanisms by which curcumin prevents cancer are thought to involve up-regulation of carcinogen-detoxifying enzymes, such as GSTs (7–9), antioxidant (10–16), and suppression of expression of the enzyme cyclooxygenase-2 (17, 18). The pharmacokinetic properties of curcumin in humans remain unexplored. In rodents, curcumin undergoes metabolic conjugation and reduction, and its disposition after oral dosing is characterized by poor systemic bioavailability (9, 17, 19, 20).

In view of the paucity of pharmacodynamic and pharmacokinetic information regarding curcumin in humans, we conducted a dose-escalation pilot study of a standardized *Curcuma* extract in patients with advanced colorectal cancer refractory to standard chemotherapy. The aims of the study were threefold. Firstly, we wished to evaluate the safety of curcumin administered p.o. as *Curcuma* extracts. Secondly, we wanted to investigate the suitability of two potential biomarkers of the pharmacological efficacy of curcumin in patients' blood leukocytes: GST activity and the levels of the adduct (M1 G) formed by the reaction of malondialdehyde with deoxyguanosine in DNA. GST enzyme activity has been shown to be up- or down-regulated in rat tissues after oral curcumin treatment, depending on the dose and route of administration (7–9). To aid the interpretation of GST activity data, patients were genotyped for GSTM1, GSTT1, and GSTP1. These represent the three major GST subclasses found in human lymphocytes, at least one of which is relevant to colorectal cancer and resistance to chemo-

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therapy (21–23). Malondialdehyde is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis via cyclooxygenase (24, 25). These two cellular processes implicated in the pathogenesis of colorectal cancer (26, 27) are inhibited by curcumin in preclinical models (17, 28). In a recent study in rats, dietary curcumin was shown to up-regulate GST activity in the liver and diminish M_G levels in colon mucosa, and these effects were accompanied by measurable tissue levels of curcumin (9). The third aim of the study described here was therefore to test the hypothesis that curcumin or products of its metabolism can be detected in blood or excreta of humans. Overall, the study was designed to define parameters that might help to optimize the clinical evaluation of curcumin in Phase I/II trials.

PATIENTS AND METHODS

Patients. The trial was approved by the local ethics committee and the United Kingdom Medicines Control Agency. Fifteen patients enrolled between September 1999 and September 2000 at the Leicester Royal Infirmary met the following eligibility criteria: (a) histologically proven adenocarcinoma of the colon or rectum for which no further conventional therapies were available; (b) measurable or evaluable disease; (c) age > 18 years; (d) WHO performance status of 0–2 and life expectancy greater than 12 weeks; (e) absolute neutrophil count ≥ 1.5 × 10^9/liter; (f) hemoglobin ≥ 10 g/dl; (g) platelets ≥ 100 × 10^9/liter; (h) aspartate aminotransferase and alanine aminotransferase < 2.5 × the upper limit of normal; (i) serum bilirubin and creatinine < 1.5 × the upper limit of normal; and (j) no previous investigational or chemotherapeutic drugs within 28 days prior to enrollment. Exclusion criteria included: (a) active chronic inflammatory or autoimmune disease; (b) active infection, including viral infection; (c) significant impairment of gastrointestinal function or absorption; (d) active peptic ulcer disease; (e) known biliary obstruction or biliary insufficiency; and (f) use of NSAIIDs within 14 days of enrollment. Patients were asked to abstain from NSAIID use and the consumption of foods containing the spice turmeric during the study period, and their general practitioners were asked not to prescribe NSAIDs. Written informed consent was obtained from each patient before enrollment. Demographic and baseline characteristics of patients are shown in Table 1. All patients were Caucasian, and all had undergone previous surgery. Three patients stopped NSAIID medication 3 weeks before enrollment.

Study Design and Treatment. P54FP was provided in soft gelatin capsules by Phytopharm plc. (Godmanchester, United Kingdom). Each capsule contained 20 mg of curcuminoids (18 mg of curcumin and 2 mg of demethoxycurcumin) suspended in 200 mg of essential oils derived from Curcuma spp. Typical constituents of Curcuma essential oil mixtures are turmerone, atlantone, and zingiberene. Chemical analysis by HPLC/mass spectrometry confirmed the content of curcuminoids. This formulation, which in the following text will be referred to as "Curcuma extract," was selected on account of the curcumin dose, which equates to dietary intake of turmeric (see below), the reproducibility of curcuminoid content, and the fact that the capsules contained extracts of Curcuma plants used in traditional Indian and Southeast Asian medicine. There were three patients per dose level. After at least a 2-h fast, patients consumed 2, 4, 6, 8, or 10 capsules once daily with water. This translates to doses of 440, 880, 1320, 1760, and 2200 mg of Curcuma extract per day containing 36, 72, 108, 144, and 180 mg of curcumin, respectively. Treatment was continued until disease progression was established or consent was withdrawn.

Clinical Measurements. Blood, urine, and feces were collected on days 1, 2, 8, and 29 and protected from light and stored at −80°C. Blood was collected before dose administration and at 0.5, 1, 2, 8, and 6 h after dose administration. Samples were collected in tubes pretreated with lithium-heparin (Sarstedt, Loughborough, United Kingdom). Full blood cell count and urea, electrolytes, liver, and bone function were measured in venous samples, and physical examination was performed before treatment and on treatment days 1, 2, 8, and 29 and monthly thereafter. Venous blood levels of the tumor markers CEA and CA19.9 were measured before treatment and after every month of treatment. Radiological assessment of target lesions was performed every 2 months by CT or magnetic resonance imaging scan, in addition to monthly chest X-rays. Blood samples for analysis of GST activity and M_G levels were collected 1 week before treatment and on days 1, 2, 8, and 29 of treatment, immediately before dosing for M_G or immediately before and 1 h after each dose for GST. Lymphocytes were separated from fresh blood using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Bucks, United Kingdom), resuspended in 1 ml of 10 mM Tris-HCl (pH 7.8), and stored at −80°C. Patients completed the European Organization for Research and Treatment of Cancer quality of life questionnaire GLQ-C30 (version 2.0) before treatment and monthly during treatment (29).

Table 1 Patient characteristics at enrollment

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<th>Daily dose of Curcuma extract (mg)</th>
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<td>60.3</td>
<td>53</td>
<td>63.7</td>
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<tr>
<td>Sites of measurable disease</td>
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<tr>
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<td>2</td>
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Chemical Analysis. Extraction of curcumin and its metabolites curcumin glucuronide, curcumin sulfate, hexahydrocurcumin, and hexahydrocurcuminol from plasma and urine was performed, and their recovery efficiency was established, as described previously (17). Curcumin and curcumin sulfate were extracted from feces with 2 parts (w/v) of acetonitrile:water (7:3). Curcuminoids were separated from other fecal constituents by C18 solid phase extraction (Varian, Walton-on-Thames, United Kingdom) and eluted from the column with acetonitrile (2 ml). The reverse-phase HPLC method with UV-visible detection used to analyze curcuminoids in extracts of plasma, urine, or feces has been reported previously (17). The limit of detection for curcumin in plasma and urine was 5 pmol/ml. The synthesis of curcumin sulfate and its identification by electrospray mass spectrometry were performed as described previously (17).

Measurement of GST Activity and MxG Levels. Glutathione and CDNB were purchased from Sigma Chemical Co. (Poole, United Kingdom). Once thawed, lymphocyte samples were sonicated for 30 s (Fisher 550 sonicator; Fisher, Pittsburgh, PA) on ice and centrifuged at 3000 × g (15 min, 4°C). Total GST activity in the supernatant was measured spectrophotometrically using glutathione and CDNB as substrates, in triplicate for each sample (30). Results were corrected for protein levels using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). The GST activity values were quoted as nmol CDNB conjugated with glutathione/min/mg lympocytic protein. The GSTM1, GSTT1, and GSTP1 genotypes were determined by PCR methods described previously (31, 32). Murine M1, M2 monoclonal antibody D10A1 was prepared as described previously (33). Antibodies and L-asparaginase were purchased from Dako (Ely, United Kingdom). M1 standards were synthesized and characterized, genomic DNA was extracted from whole blood, and leukocytic M1 adduct levels were analyzed by immunoslot blot in triplicate as described previously (34). Discrepancies in the amount of DNA/slot were corrected for by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry (9). The detection limit for M1 was 5 adducts/10⁸ nucleotides. The assay had been validated previously by collaborating laboratories at the University of Leicester and the Vanderbilt Cancer Center by exchange of samples.

Statistical Evaluation. GST and M1 values were subjected to a balanced repeated measure ANOVA and linear regression analysis using Minitab (version 10.2) software package. Plots of residuals were used to ensure that variances were homogeneous and that the residuals had a normal distribution.

RESULTS

Tolerability of Oral Curcuma Extract. Patients with advanced colorectal cancer ingested P54FP capsules once daily for up to 4 months at doses between 440 and 2200 mg of Curcuma extract containing between 36 and 180 mg of curcumin. The treatment was well tolerated at all dose levels, and there was no dose-limiting toxicity. Two types of adverse events, both gastrointestinal, were possibly related to Curcuma consumption. One patient on 1320 mg of Curcuma extract daily experienced nausea during the first month of treatment (National Cancer Institute toxicity grade 1), which resolved spontaneously despite continuation of treatment. Two patients (one each on 880 and 2200 mg of Curcuma extract daily) developed diarrhea (National Cancer Institute grades 2 and 1, respectively) 4 months and 1 month into treatment, respectively. Both patients withdrew from the study before the cause of the diarrhea could be investigated.

Pretreatment GST and M1 Levels in Relation to GST Polymorphisms. Lymphocytic total GST activity and leukocytic M1 levels differed substantially between patients (Figs. 1 and 2). Patients were genotyped for GST isoenzymes GSTM1, GSTP1 and GSTT1. Two-thirds of the patients lacked GSTM1, slightly more than the 40-60% proportion expected in healthy Caucasians (21), with an even distribution across the five dose levels. In patients who displayed the null genotype for GSTM1, pretreatment levels of leukocytic M1 were 7.6 ± 4.3 adducts/10⁷ nucleotides, 74% higher than those in patients expressing GSTM1, in whom adduct levels were 4.3 ± 2.6 adducts/10⁷ nucleotides (P < 0.001 by ANOVA). Two patients were null for GSTT1. Their pretreatment levels of leukocytic M1 (mean levels pooling triplicate readings from both time points, 5.8 ± 1.6 adducts/10⁷ nucleotides) were marginally lower than those in patients expressing GSTT1 (6.6 ± 4.3 adducts/10⁷ nucleotides; P = 0.02 by ANOVA). Leukocytic levels of M1 did not correlate with total GST activity, active smoking status (n = 2), vegetarianism (n = 2), or age.

Biological Effects of Oral Curcuma Extract. In patients taking 440 mg of Curcuma extract (36 mg of curcumin) daily, lymphocytic GST activity decreased gradually with time from a pretreatment GST value of 64 ± 19 nmol/min/mg protein.
DISCUSSION

The study presented here constitutes the first clinical evaluation of a standardized *Curcuma* extract in patients with cancer including pharmacodynamic and pharmacokinetic measurements. The results allow three conclusions, which will help to optimize the design of future clinical trials of curcumin or *Curcuma* extracts: (a) oral administration of *Curcuma* extract for several months at doses of up to 2.2 g daily (equivalent to 180 mg of curcumin) appears safe in the framework of this Phase I study; (b) the systemic bioavailability of p.o. administered curcumin is low in humans; and (c) *Curcuma* extract may cause clinical benefit in patients with advanced refractory colorectal cancer.

Our first conclusion regarding the apparent safety of *Curcuma* extracts is consistent with previous reports of clinical studies of curcumin and turmeric. Soni and Kuttan (35) treated 10 volunteers with 500 mg of curcumin daily for 7 days and failed to observe clinical toxicity. Two clinical trials designed to study the efficacy of curcumin as an anti-inflammatory agent in the treatment of arthritis or postoperative inflammation found that daily doses of 1.2–2.1 g of curcumin for 2–6 weeks did not cause adverse effects (36, 37). In a pilot study published in abstract form (38), tablets of turmeric extract containing 99.8% curcumin did not cause any treatment-related toxicity at doses as high as 8 g/day. Furthermore, a single dose of 50–200 mg of micronized curcumin formulated as capsules or sachets was administered to 18 volunteers without causing significant tox-
iciety. Clinical trials of oral curcumin incorporating larger subject populations will be required to establish the safety of chronic administration. Although certain communities in the Indian subcontinent consume up to 1.5 g of dietary turmeric per person per day; curcumin constitutes only 2–8% of most turmeric preparations (39). The acceptable daily intake of curcumin as an additive has been defined by the WHO as 0–1 mg/kg body weight (40). Thus the largest dose administered in the study presented here (2.2 g of Curcuma extract, containing 180 mg of curcumin) exceeds that of dietary consumption.

Our finding that curcumin was detectable only in the feces of patients and not in plasma, blood cells, or urine is in keeping with the low systemic bioavailability of p.o. administered curcumin seen in rodents (9, 17, 19, 20) and suspected in humans (38). After a single oral dose of 2 g, curcumin levels were transiently detectable in the serum of healthy volunteers (41). In that study, coinigestion of curcumin with the pepper constituent 1-piperonylpiperidine, which is thought to inhibit xenobiotic glucuronidation, appeared to increase curcumin serum AUC by a factor of 20. The presence of curcumin sulfate in the feces of one patient at the highest dose level described here is consistent with the suspicion that curcumin can undergo metabolic conjugation in the gut (42). We are currently testing the hypothesis that sulfation is the major biotransformation route of the curcumin molecule catalyzed by intestinal tissue.

Two potential biomarkers of the systemic efficacy of curcumin were evaluated in the pilot study described here. Lymphocytic GST activity decreased with time in the three patients who received the lowest dose level of Curcuma extract. This decrease may have been associated with the treatment, but in light of the small number of patients studied and the fact that GST activity was not decreased in patients on higher dose levels, the interpretation of this observation has to be tentative. Rats fed dietary curcumin at approximately 250 mg/kg body weight and above were found to have decreased hepatic GST activity compared to controls, and competitive enzyme inhibition by the curcumin molecule was thought to be responsible (7). It is unlikely that this observation can be used to rationalize the decline observed in our patients because the dose used in the rats was more than 60-fold higher than that given to the patients. Lymphocytic GST activity, as measured by the CDNB assay, has been shown to be independent of age and gender (43) and constant within subjects, as borne out by measurements on at least three occasions over a 2–4-week period in normal individuals and those at increased risk of developing colorectal cancer (44). The observations made in the study reported here propose similar consistency for patients with advanced cancer. Whether or not lymphocytic GST activity correlates with colon mucosal GST levels in patients with colon cancer, as was demonstrated in individuals at risk of developing colon cancer (44), remains to be established.

This study provides the first description of leukocytic M,G levels in patients with colorectal cancer. The lower levels shown in Fig. 2 are comparable with those reported previously in healthy volunteers, whereas the highest levels resemble those seen in humans on pro-oxidant diets (45). M,G adduct levels were unaffected by Curcuma consumption. It is conceivable that higher doses of curcumin, which furnish measurable plasma curcumin concentrations, are required to elicit an antilipid peroxidative effect in the blood. Nevertheless, the intradividual reproducibility over time of M,G adduct levels supports the potential suitability of this adduct as a biomarker of the systemic effects of curcumin or other chemopreventive antioxidants. The putative link between the GSTM1 null genotype and elevated leukocytic M,G adduct levels observed in the patients in this trial is congruous with associations reported previously between GSTM1 genotype and levels of aflatoxin B1-induced DNA adducts (46) but is the first suggestion of such an association for an adduct formed by an endogenous product of lipid peroxidation.

One-third of the patients in this study experienced stable disease for 3 months or longer, and in one additional patient, Curcuma extract may have been linked to a decrease in venous tumor marker level and abatement of progression of the primary colon tumor without a cytostatic effect on liver metastases. The possibility that patients with colorectal cancer may benefit from consumption of Curcuma extract merits evaluation at higher dose levels and ultimately within the framework of larger studies incorporating control groups.

In conclusion, despite the lack of reproducible effects of Curcuma extracts on the biomarkers studied, this pilot study of Curcuma extract in patients with colorectal cancer provides information that might help optimize the design of the future clinical evaluation of curcumin. Doses of up to 2.2 g of Curcuma extract (containing 180 mg of curcumin) per day can be administered to patients with cancer for up to 4 months, and in this pilot study, such treatment was safe. Clinical trials of Curcuma extracts as potential cancer chemopreventive agents should focus on the effects of such doses in target tissues, particularly colon epithelium. Moreover, because consumption of Curcuma extract was not detrimental to patients with advanced cancer, future trials of Curcuma extracts as potential cancer chemotherapeutic agents should study the systemic effects of higher dose levels. Leukocytic GST activity and M,G levels merit further exploration as potentially suitable biomarkers of pharmacological efficacy in this regard.

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1900 Study of Oral Curcuma in Patients with Colorectal Cancer


Effects of Dietary Curcumin on Glutathione $S$-Transferase and Malondialdehyde-DNA Adducts in Rat Liver and Colon Mucosa: Relationship with Drug Levels

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ABSTRACT

Curcumin prevents colon cancer in rodent models. It inhibits lipid peroxidation and cyclooxygenase-2 (COX-2) expression and induces glutathione $S$-transferase (GST) enzymes. We tested the hypothesis that 14 days of dietary curcumin (2%) affects biomarkers relevant to cancer chemoprevention in the rat. Levels of inducible COX-2, as reflected by prostaglandin $E_2$ production by blood leukocytes, were measured ex vivo. Total GST activity and adducts of malondialdehyde with DNA (M$_2$G), which reflect endogenous lipid peroxidation, were measured in colon mucosa, liver, and blood leukocytes. Curcumin and its metabolites were analyzed by high-performance liquid chromatography in plasma, and its pharmacokinetics were compared following a diet containing 2% curcumin versus intragastric (i.g.) administration of curcumin suspended in an amphiphilic solvent. The curcumin diet did not alter any of the markers in the blood but increased hepatic GST by 16% and decreased colon M$_2$G levels by 36% when compared with controls. Administration of carbon tetrachloride during the treatment period increased colon M$_2$G levels, and this increase was prevented by dietary curcumin. Dietary curcumin yielded low drug levels in the plasma, between 0 and 12 nm, whereas tissue concentrations of curcumin in liver and colon mucosa were 0.1–0.9 nmol/g and 0.2–1.8 $\mu$mol/g, respectively. In comparison with dietary administration, suspended curcumin given i.g. resulted in more curcumin in the plasma but much less in the colon mucosa. The results show that curcumin mixed with the diet achieves drug levels in the colon and liver sufficient to explain the pharmacological activities observed and suggest that this mode of administration may be preferable for the chemoprevention of colon cancer.

INTRODUCTION

Cancer mortality rates in the developed world have risen throughout most of this century, and it is already the leading cause of death in some Western countries (1, 2). This observation has engendered much research activity aimed at the identification of cancer chemopreventive agents, especially substances derived from the diet (3). Turmeric, the dried ground rhizome of the perennial herb Curcuma longa, is an example of one such agent. This spice is consumed in the diet in quantities up to 4 g/adult/day in some countries (4), which also appear to have low incidence rates of colorectal cancer (1). Turmeric contains curcuminoids and essential oils. Curcumin (diferuloylmethane), a low molecular weight polyphenol and the major curcuminoid in the plant, is regarded as the constituent with the highest biological activity. Curcumin has been shown to inhibit tumor formation in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (5). Mechanisms by which curcumin causes cancer chemoprevention are thought to involve antioxidation (6), inhibition of kinases (7), interference with the activity of transcription factors such as nuclear factor-$\kappa$B and activator protein-1 (8), and suppression of expression of the enzyme COX-2 (9).

In a program of preclinical work designed to help optimize the clinical evaluation of curcumin as a colon cancer chemopreventive agent, we tested the hypothesis that curcumin in the diet alters biomarkers of its chemopreventive efficacy. Three biomarkers were selected to represent possible mechanisms of the chemopreventive activity of curcumin in vivo. These were total GST activity, DNA adducts formed by MDA, and inducible COX-2 expression as reflected by PGE$_2$ production. Induction of GSTs, phase II enzymes that detoxify certain carcinogens, is regarded as a potential mechanism of blockade of the early stages of carcinogenesis (3). Such induction is a property...
of several agents with chemopreventive activity in preclinical models, including curcumin (10) and the broccoli constituent sulforaphane (11). MDA is a naturally occurring product of lipid peroxidation (12), which is also formed during prostaglandin biosynthesis via COX (13), two enzymatic processes that have been implicated in the pathogenesis of a number of cancers, especially colon cancer (14–16). PGE₂ is a product of COX-2, the isoenzyme of COX induced during infection, inflammation, and malignant transformation. Inhibition of COX-2 is thought to be an important mechanism of chemoprevention, exhibited by nonsteroidal anti-inflammatory drugs (14) and dietary agents such as curcumin (9). MDA reacts with DNA under physiological conditions to form adducts, predominantly with deoxyguanosine (M₆G). Accumulated DNA damage may be important in the etiology of many cancers, and such damage may be reflected by exocyclic DNA adducts such as M₆G (17). M₆G has been implicated in the induction of G→T transversions by MDA and is considered a potentially useful “dosimeter” of MDA-induced DNA modification (18). M₆G levels have been described in malignant and nonmalignant human colon cells in vitro and in leukocytes, pancreas, breast, and liver in healthy volunteers (12); indeed they are comparable with levels of DNA adducts formed by exogenous carcinogens such as polycyclic hydrocarbons.

The ability of curcumin to prevent malignancies in the colorectal tract in rodents has been particularly well documented (7, 19, 20). It has also been shown to inhibit lipid peroxidation (21) and carbon tetrachloride-mediated hepatotoxicity (22, 23). We therefore aimed to test the hypothesis that dietary curcumin affects GST and M₆G levels in the intestinal mucosa and prevents increases in M₆G levels when lipid peroxidation is induced in this tissue by CCl₄. For comparison, levels of GST and M₆G were also investigated in liver and blood leukocytes. Because COX-2 is not expressed significantly in normal tissues without provocation, an ex vivo model was used to study its inducibility in peripheral blood indirectly. To be able to rationalize potential pharmacodynamic changes and interpret them in terms of efficacious drug levels, curcumin and its glucuronide or sulfate conjugation products were determined in intestinal mucosa, plasma, and liver. Studies in rodents suggest that curcumin has poor systemic bioavailability when given p.o. and is cleared rapidly from the plasma when administered i.v. (24), but overall the information on its pharmacokinetic behavior is scarce. Whereas in cancer intervention studies using curcumin it has generally been administered admixed in the diet, studies of its pharmacokinetics and metabolism have used i.g. gavage or non-oral routes of administration. In the light of the difficult pharmaceutical properties of curcumin, i.e., its lipophilicity and susceptibility to rapid metabolism, we also tested the hypothesis that drug and metabolite levels after i.g. gavage are representative of those following curcumin ingestion in the diet.

**MATERIALS AND METHODS**

**Reagents.** Curcumin was purchased from Apin Chemicals Ltd. (Abingdon, United Kingdom). Analysis by HPLC/mass spectrometry established that the material contained 91% curcumin and 9% desmethoxycurcumin. CCl₄, corn oil, glutathione, CDNB, glycerol formal (consisting of 60% 5-hydroxy-1,3-dioxane and 40% 4-hydroxymethyl-1,3-dioxalone) and Cre mopore were purchased from Sigma Chemical Co. (Poole, United Kingdom). Murine M₆G monoclonal antibody D10A1 was prepared as described previously (25). Antimurine horseradish peroxidase antibody was purchased from Dako (Ely, United Kingdom). M₆G standards were synthesized and characterized as described previously (26).

**Treatments.** Female F344 rats (6-weeks of age; 160–180 g), obtained from Charles River UK Ltd. (Margate, United Kingdom) had access to water and a standard RMI diet (Special Diet Services) ad libitum. Rats were kept in groups of four per cage at 20°C on a 12-h light/dark cycle. For dietary administration, curcumin and corn oil were mixed with the diet to furnish 2% for each. This level of curcumin in the diet, which rats received for 14 days, has been shown previously to prevent gastrointestinal tract tumors in several rodent models of chemically induced carcinogenesis (5, 10). Control animals received a diet containing corn oil. CCl₄ was dissolved in corn oil (2 ml/kg) and administered by i.g. gavage at 0.5 ml/kg on day 10 after commencement of dietary curcumin intake. This dose of CCl₄ has been shown previously to raise liver M₆G levels (18). Control animals received the equivalent dose of corn oil.

In the pharmacokinetic study, administration of dietary curcumin was compared with that of an i.g. bolus. For administration by gavage, curcumin was suspended and partially dissolved in a mixture of glycerol formal:cremophore:water (5:2:2), which in preliminary experiments was found to yield a suitable formulation in terms of acceptable viscosity and stability of suspension. This formulation was administered at 500 mg curcumin/kg by i.g. gavage once only or daily for 7 consecutive days. Control animals received the excipient mixture only. Tissue samples were collected 30 min postdose, a time point at which in preliminary experiments curcumin levels had been found to be maximal. For an optimal comparison of this administration mode with dietary curcumin, we exploited the fact that feeding habits of rats are subject to a diurnal cycle with two eating peaks, one of which occurs at around 8:00 p.m. (27). Rats were deprived of food for 6 h, commencing at 1:00 p.m. They then received the curcumin-containing diet for 3 h, coinciding with the eating peak. Subsequently, food was withheld for ~30 min, after which blood and tissue samples were collected. This “starvation-refeeding” protocol was performed either in untreated animals or in rats that had received the curcumin diet continually for 7 days.

At the end of the feeding period, or subsequent to i.g. administration, rats were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture. Blood was placed in heparinized tubes and plasma obtained by centrifugation (1100 × g at 4°C for 25 min). The large intestine was flushed with PBS and dissected out. Cytosol was prepared by standard procedures (28), and blood leukocytes were isolated using Ficoll-Paque Plus (Amersham Pharmacia

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Biotech, Buckinghamshire, United Kingdom). Histological examination ensured accurate scraping of colonic mucosa only. Blood and tissue samples were protected from light, frozen in liquid nitrogen, and stored at −80°C until pharmacodynamic and pharmacokinetic analyses were performed.

**Pharmacodynamic Analyses.** Total GST activity of cytosol samples was measured spectrophotometrically using glutathione and CDNB (29). Results were corrected for protein levels using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). Extraction of genomic DNA and analysis of M,G adduct levels by immunoslot blot was performed as described previously (30). Discrepancies in the amount of DNA in each slot were corrected by staining the nitrocellulose filter with propidium iodide and performing UV light densitometry. The detection limit for M,G was five adducts per 10^6 nucleotides. The model for assessing inducibility of COX-2 ex vivo was based on published methods and has been well established in clinical studies as well as preclinical models (31, 32). Aliquots (0.5 ml) of fresh blood were incubated with acetylsalicylic acid (200 μM) for 30 min at 37°C to inactivate platelet COX-1 irreversibly. LPS (10 μg/ml) was added to half the samples and mixed well, and samples were reincubated for 24 h. Plasma was separated by centrifugation and stored at −80°C. PGE_2 in these plasma samples was measured by competitive enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI), with a detection limit of ∼30 pg/ml plasma.

**Analysis of Curcumin and Its Conjugates.** Curcumin and its metabolites were measured as described before (24). Plasma and tissues were extracted with twice the volume of ethyl acetate, and the organic layer was evaporated under nitrogen. Extraction efficiencies from plasma for curcumin, curcumin glucuronide, and curcumin sulfate determined by HPLC (see below) at 0.1 μg/ml were 92 ± 7, 45 ± 10, and 49 ± 9% (mean ± SD, n = 6), respectively; the extraction efficiencies at 40 μg/ml were very similar to these values. Extraction efficiencies from liver and colon mucosal scrapings were not significantly different from those obtained in plasma. The reversed-phase HPLC method for detection and quantitation of curcumin and its conjugates used a Symmetry Shield RP 18 column (150 × 3.9 mm; particle size, 5 μm; Waters) and a Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410). Detection of curcumin, curcumin sulfate, and curcumin glucuronide was achieved at 420 nm. For the detection of products of curcumin reduction, the detector was switched to 280 nm.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Curcumin-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>634 ± 66^a</td>
<td>860 ± 72^a</td>
</tr>
<tr>
<td>Colon mucosa</td>
<td>84 ± 13</td>
<td>69 ± 16</td>
</tr>
<tr>
<td>Blood lymphocytes</td>
<td>20 ± 4</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>

^a Animals were exposed to curcumin (2%) in the diet for 14 days.
^b Values, expressed as nmol/min/mg protein using CDNB as substrate, are the mean ± SD of eight separate animals. For experimental details see "Materials and Methods."

^c The difference between control and treated animals is significant (P < 0.01, by ANOVA).

**RESULTS**

**Pharmacodynamic Effects of Dietary Curcumin.** Rats were fed a diet supplemented with 2% curcumin for 2 weeks. On the basis of food consumption, the approximate daily dose ingested was 1.2 g curcumin/kg. Dietary curcumin at this dose did not affect the animals’ body weight gain. Levels of GST activity in the liver of curcumin-fed rats were elevated by 36% over those in control animals (Table 1). GST levels in colon mucosa and lymphocytes were marginally decreased by curcumin; however, this difference was not significant. Levels of M,G adducts in colon mucosa of rats which received curcumin were moderately, but significantly, lower than those in control rats (Fig 1). M,G adduct levels in leukocytes and liver of curcumin-fed rats were also slightly decreased as compared with controls, but these differences were not significant. Treatment of rats with CCl_4 increased levels of M,G adducts in liver and colon mucosa by 49 and 25%, respectively, over control values (Fig 1). Dietary curcumin attenuated this increase in the liver and completely prevented it in the colon.

To study whether dietary curcumin led to altered COX-2 expression in blood cells, the concentration of PGE_2 in plasma was determined after induction with LPS added in vitro. LPS increased blood PGE_2 levels ∼3-fold, but curcumin feeding failed to interfere with this increase (result not shown).

**Levels of Curcumin after Dietary Administration.** Plasma, colon mucosa, and liver from rats that had received curcumin in the diet as described above for 2 weeks were analyzed for the presence of curcumin. Curcumin and its metabolites, curcumin glucuronide and curcumin sulfate, could not be detected in the plasma obtained by cardiac puncture or from the hepatic portal vein. Levels of curcumin were 1.8 ± 0.8 μmol/g tissue in the colon mucosa and 0.8 ± 0.3 nmol/g in the liver. Curcumin was also present in the feces (8.6 ± 0.6 μmol/g dried feces). Curcumin glucuronide or curcumin sulfate was not detected in either tissues or feces.

**Pharmacokinetic Comparison of Modes of Administration.** To compare the availability of curcumin administered with the diet or suspended in an amphiphilic solvent and adminis-
Fig 1  Effect of dietary curcumin on M, G adduct levels in liver, colon mucosa, and blood leukocytes. ■, control animals; □ and □, animals that received dietary curcumin (2%) for 14 days; □ and □, animals that received carbon tetrachloride (0.5 ml/kg) via the i.g. route on the 10th day of the study diet. Values are means of eight animals; bars, SD. * and **, the difference between control and curcumin-treated animals (P < 0.01, by ANOVA) or between animals that received carbon tetrachloride either with or without curcumin (**, P < 0.005, by ANOVA) is significant. For experimental details, see "Materials and Methods."

Table 2  Curcumin and curcumin conjugates in the plasma of rats that received curcumin in the diet or by the i.g. route

<table>
<thead>
<tr>
<th>Dosimetry schedule</th>
<th>Curcumin</th>
<th>Curcumin glucuronide</th>
<th>Curcumin sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short feeding</td>
<td>&lt;10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Single i.g.</td>
<td>30 ± 9</td>
<td>56 ± 36</td>
<td>134 ± 100</td>
</tr>
<tr>
<td>7-day feeding</td>
<td>12 ± 5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Repeated i.g.</td>
<td>65 ± 28</td>
<td>638 ± 146</td>
<td>360 ± 160</td>
</tr>
</tbody>
</table>

<sup>2</sup> Rats received curcumin as an i.g. bolus (500 mg/kg) once ("Single i.g.") or daily for 7 consecutive days ("Repeated i.g."); or with the diet (2%) for 3 h after a period of starvation ("Short feeding"), or after a starvation-refeeding regimen after unlimited access to a curcumin-containing diet for 7 days ("7-day feeding"). The dose of curcumin when given with the diet, as calculated by weighing food removal, was between 200 and 400 mg/kg. For details of curcumin administration and its measurement, see "Materials and Methods."

<sup>6</sup> Values are mean ± SD from three to four animals.

Curcumin and its conjugates were observed in the plasma only when the agent was given via the i.g. route (Table 2). Repeated i.g. administration for 7 days led to an increase in plasma levels of curcumin and its conjugates as compared with single i.g. administration. Curcumin levels were doubled, and levels of curcumin sulfate and curcumin glucuronide were 2.7 and 11 times higher than those seen after single i.g. administration. In contrast, plasma levels of curcumin after its consumption in the diet for a week, at the end of which rats were subjected to the starvation-refeeding regime, did not differ substantially from those measured after short-term dietary consumption. The variation between animals in colon mucosal curcumin levels (Table 3) after dietary consumption was very high, probably reflecting considerable differences in food intake between individual animals during the 3-h period of refeeding. Curcumin levels in the colon mucosa after short-term dietary consumption were 164 times higher than those seen after single i.g. bolus; after dietary consumption for 1 week, they were 27 times higher than after the last of seven daily i.g. bolus doses (Table 3). Liver levels of curcumin were ~0.1 nmol/g, irrespective of route of administration, and there was little difference in liver levels between single or repeated administration via the diet or gavage.

DISCUSSION

The results of this study allow two conclusions that may help to optimize clinical trials of curcumin as a cancer chemopreventive agent: (a) the bioavailability of curcumin in blood and tissues is dramatically affected by the way in which it is p.o. administered; and (b) colon mucosa and liver are pharmacological targets of dietary curcumin.

The first conclusion is based on the observed differences in levels of curcumin and its conjugates in plasma and tissues after the two administration modes. Dietary curcumin elicited concentrations of the drug in the colon mucosa of between 0.3 and 1.8 μmol/g, whereas plasma levels were around the limit of detection. Curcumin suspended in a solvent mixture and given by i.g. bolus furnished levels of drug in the plasma that were 3–6-fold higher than those seen after dietary administration. Curcumin metabolites were detectable in the plasma only after i.g. administration. Conversely, colon mucosal levels of curcumin after i.g. bolus were only a fraction of those observed after dietary administration.

Curcumin exerts its effects on growth, COX-2 expression,
and transcription factor activity in cells in vitro at concentrations of 5 μM or above (9). The colon mucosal level of curcumin after 14 days feeding observed in this study, 1.8 μmol/g, is more than 300-fold higher than the minimal concentration shown to be active in vitro. This result demonstrates that dietary administration of curcumin can produce pharmacologically relevant drug concentrations in colon mucosa. Although the dose used in this study, 2% in the diet, has been used frequently in rodent intervention studies (5, 10, 33), it is at least 10 times higher than the highest estimated daily human intake of curcumin as a dietary constituent (4) and does not reflect normal dietary use. In recent intervention studies, dietary levels of 0.2 and 0.1% curcumin have been demonstrated to protect rodents from colon cancer induced by azoxymethane (7, 19, 20) and to reduce polyp numbers in the Min mouse (34), a model of the genetic defect found in human familial adenomatous polyposis. Our results therefore intimate that dietary curcumin at doses considerably lower than those used here might yield pharmacologically efficacious levels in the colon mucosa and perhaps also in the liver.

Intriguingly, curcumin glucuronide and curcumin sulfate were not detected in plasma or tissues after its administration in the diet, whereas they were the major drug-derived species present in the plasma after i.g. administration. In a recent report, a small amount of a curcumin reduction product, tetrahydrocurcumin, was found in plasma samples from rats fed a curcumin-containing diet (1%) subsequent to treatment of plasma with enzymes that hydrolyze xenobiotic glucuronides and sulfates (35). In contrast, products of curcumin reduction were not unequivocally detected in plasma after dietary curcumin given for 14 days in the experiments described here, although hexahydrocurcumin and hexahydrocurcuminol were identified by mass spectrometry in the bile. Overall, this part of the study suggests that the pharmacokinetic behavior of curcumin after administration of an i.g. bolus of curcumin in suspension is clearly unrepresentative of that of curcumin mixed into the diet. This finding is important because, although in intervention studies curcumin is generally given as a constituent of the diet, studies of its pharmacokinetics and metabolism have been performed mostly with the drug formulated in suspension, using DMSO, aqueous carboxymethyl cellulose solution, or arachis oil as solvents, and administered as an i.g. bolus or via the i.p. route (36–39). The results presented here suggest that oral curcumin be advocated in the chemoprevention of malignancies remote from the liver or gastrointestinal tract, improvement of its oral bioavailability might be necessary, perhaps by formulating it as a solution.

Our second conclusion is borne out by the findings that dietary curcumin elevated hepatic GST levels, reduced colon mucosal M,G adduct levels, and decreased the elevation of M,G adduct levels elicited by a powerful lipid peroxidative stimulus in the liver and colon mucosa. These potentially beneficial effects correlated with curcumin levels of 1.8 μmol/g in the colon mucosa and 0.8 nmol/g in liver. The high concentrations of curcumin in the colon mucosa described here are consistent with the outcome of intervention studies in preclinical rodent models of colon cancer (7, 19, 20, 34). Although the decrease in M,G levels in colon mucosa was modest, it underlines in principle that curcumin supplementation of the diet can achieve drug concentrations sufficient to decrease levels of DNA adducts formed as a corollary of lipid peroxidation. Curcumin glucuronide and curcumin sulfate were not found in liver or colon mucosa after dietary administration of curcumin, strongly suggesting that parent curcumin rather than either conjugate affects GST activities and M,G adduct levels in vivo.

The levels of M,G adducts in rat liver measured here were approximately three times higher than those described previously in Sprague Dawley rats and are more analogous to background levels found in normal human liver (18). Differences may be related to the age, sex, and strain of the animals used, or fat composition of the diets used. M,G adduct levels in rat leukocytes and colon mucosa have not been documented previously. CCl₄ has been shown to increase MDA and M,G adduct levels in rat liver (18), and increases in hepatic MDA levels have been attenuated by feeding rats 100 mg/kg curcumin for 4 weeks (23). The results described here extend these findings, because they show that CCl₄ also engenders M,G adducts in the colon mucosa, and that this increase can be prevented by dietary curcumin. These findings are pertinent in the light of a recent study in which oxidative DNA damage linked to lipid peroxidation was detected in biopsies from normal human colon (40). The M,G-lowering effect of curcumin provides a tentative rationale for the regular use of dietary curcumin in the protection of the colon mucosa against oxidative damage, perhaps in premalignant conditions such as ulcerative colitis.

Liver GST activity was raised over controls after curcumin ingestion in the diet. The effects of curcumin on GST and its expression are complex and may involve competitive enzyme inhibition (41) as well as indirect enzyme induction (42). Our finding that 14 days of dietary curcumin induces GST activity in the liver is consistent with an earlier study of the same dose of curcumin in diet and water fed to 8-week-old mice (10). Similarly, mice that received curcumin dissolved in aqueous sodium carboxymethylcellulose via i.g. gavage for 15 days at a dose approximately one-fifth of that used here were found to have significantly higher GST levels in liver compared with controls (41). In contrast, in a more recent study of 8-week-old Sprague Dawley rats, curcumin dissolved in corn oil given daily for 14 days via the i.g. route at various doses failed to induce hepatic GST at doses >6% of the dietary dose used here (43). This discrepancy may relate to differences between the studies in rodent species and strain, age of the animals, dose of curcumin, and its route of administration. Compatible with earlier rodent studies of dietary curcumin (10, 44), we failed to detect any significant alteration in GST activity in the colonic mucosa.

In cells in vitro, we have shown previously that curcumin inhibits COX-2 expression by a mechanism involving interference with nuclear factor-kB activation and inhibition of the IkB kinase complex (9). In the study described here, PGE₂ production induced ex vivo as an indicator of leukocyte COX-2 activity was not affected by dietary curcumin. The fact that curcumin administered in the diet did not affect COX-2 inducibility, GST activity, or M,G levels in rat blood leukocytes is consistent with the finding that this mode of administration furnished extremely low levels of parent curcumin in the plasma.

Unpublished result.
In conclusion, the results presented above show that dietary administration of curcumin to rats produces pharmacologically active levels of unmetabolized curcumin in the colon mucosa and liver, capable of decreasing M$_G$ levels and elevating GST activity. These effects may contribute to cancer chemoprevention. The results also suggest that dietary admixture may be the preferable mode of administration for curcumin in the chemoprevention of colon cancer. The chemopreventive efficacy of oral curcumin in the colorectum and the liver merits clinical evaluation.

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Characterization of Metabolites of the Chemopreventive Agent Curcumin in Human and Rat Hepatocytes and in the Rat in Vivo, and Evaluation of Their Ability to Inhibit Phorbol Ester-induced Prostaglandin E$_2$ Production

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ABSTRACT

Curcumin, the yellow pigment in turmeric, has been shown to prevent malignancies in a variety of tissues in rodents, especially in the intestinal tract. Pharmacological activities of curcumin in cells in situ germane to chemoprevention, such as inhibition of expression of cyclooxygenase-2 (COX-2), require drug concentrations in the $10^{-5}$-$10^{-4}$ M range. The systemic bioavailability of curcumin is low, so that its pharmacological activity may be mediated, in part, by curcumin metabolites. To investigate this possibility, we compared curcumin metabolism in human and rat hepatocytes in suspension with that in rats in vivo. Analysis by high-performance liquid chromatography with detection at 420 and 290 nm permitted characterization of metabolites with both intact diferoylmethane structure and increased saturation of the heptatriene chain. Chromatographic inferences were corroborated by mass spectrometry. The major metabolites in suspensions of human or rat hepatocytes were identified as hexahydrocurcumin and hexahydrocurcuminol. In rats, in vivo, curcumin administered i.v. (40 mg/kg) disappeared from the plasma within 1 h of dosing. After p.o. administration (500 mg/kg), parent drug was present in plasma at levels near the detection limit. The major products of curcumin biotransformation identified in rat plasma were curcumin glucuronide and curcumin sulfate whereas hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide were present in small amounts. To test the hypothesis that curcumin metabolites resemble their progenitor in that they can inhibit COX-2 expression, curcumin and four of its metabolites at a concentration of 20 µM were compared in terms of their ability to inhibit phorbol ester-induced prostaglandin E$_2$ (PGE$_2$) production in human colonic epithelial cells. Curcumin reduced PGE$_2$ levels to preinduction levels, whereas tetrahydrocurcumin, previously shown to be a murine metabolite of curcumin, hexahydrocurcuminol, and curcumin sulfate, had only weak PGE$_2$ inhibitory activity, and hexahydrocurcuminol was inactive. The results suggest that (a) the major products of curcumin biotransformation by hepatocytes occur only at low abundance in rat plasma after curcumin administration; and (b) metabolism of curcumin by reduction or conjugation generates species with reduced ability to inhibit COX-2 expression. Because the gastrointestinal tract seems to be exposed more prominently to unmetabolized curcumin than any other tissue, the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent.

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major yellow pigment extracted from turmeric, the powdered rhizome of the herb Curcuma longa. Turmeric is a spice used extensively in curries and mustards as a coloring and flavoring agent. Consumption of turmeric and curcumin has been associated with a plethora of beneficial effects on human health; prominent among them are anti-inflammatory and cancer chemopreventive activities (1). Curcumin has been shown to inhibit tumor formation in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (for review see Ref. 2). Especially noteworthy are results of a number of recent studies of curcumin in colon cancer chemoprevention models in rodents. Curcumin (0.2% w/v) in the diet inhibited the development of azoxymethane-induced colonic adenocarcinomas in rats irrespective of whether the compound was administered during the initiation/postinitiation (3) or the promotion/progression stages of the disease (4). At dietary levels of 0.1%, curcumin caused a 64% reduction in adenoma formation in the intestine of Min mice, which harbor the defect in the adenomatous polyposis coli gene underlying familial adenomatous polyposis in humans (5). Curcumin has shown a variety of biological activities that might explain its chemopreventive action. These activities include antioxidation (6, 7), suppression of c-Jun/AP-1 activation (8), inhibition of prostaglandin biosynthesis (9), and inhibition of the activity and expression of the enzyme COX (10).

We have recently reported that curcumin interferes with the expression of the COX isoenzyme COX-2 and that this interference is probably linked to its ability to block activation of the transcription factor nuclear factor κB at the level of the NIK/IKKα/β signaling complex (11). In cell incubations in vitro these effects of curcumin were observed in the $10^{-5}$-$10^{-4}$ M concentration range. The bioavailability of curcumin in rodents has been shown to be low (12, 13). In a recent study, an oral dose of 1 g/kg administered to mice yielded a peak plasma level of only 0.5 µM (13). There is preliminary evidence derived from a clinical pilot study that suggests that the systemic availability of curcumin is also poor in humans, because oral doses of 4–8 g generated peak plasma levels of as little as 0.41–1.75 µM (14). These findings cast doubt on the assumption that consumption of curcumin as a drug or food constituent furnishes levels of compound in blood and tissues sufficient to elicit biological effects associated with chemoprevention, and they render rational selection of a potentially chemopreventive dose difficult. It is conceivable that curcumin is biotransformed to species that are responsible for, or contribute to, its chemopreventive efficacy. The metabolism of curcumin in humans is poorly understood. In rodents its major metabolic pathway involves successive reduction via dihydrocurcumin and tetrahydrocurcumin to hexahydrocurcumin (see Fig. 1) and conjugation of mainly tetrahydrocurcumin and hexahydrocurcumin with glucuronic acid (13, 15). The liver is the primary organ that generates metabolites from drugs and other xenobiotics. Early studies suggest that curcumin undergoes extensive metabolism in rat hepatocytes in vitro, although the metabolic products were not identified (16). The rat has served extensively as an experimental model in the evaluation of the ability of curcumin to prevent carcinogen-induced cancer (3, 4).
and rats were incubated with curcumin, and their metabolites were tested. We hypothesized that curcumin is biotransformed similarly by human and rat liver. To that end, hepatocytes obtained from humans and rats were incubated with curcumin, and their metabolites were identified. Curcumin was also administered to rats via i.v. and p.o. routes, and its plasma metabolites were compared with those found in suspensions of liver cells. Finally, to investigate whether the identified metabolites possess pharmacological properties germane to chemoprevention, we compared their ability with that of curcumin to inhibit phorbol ester-induced COX-2 expression in human colon cells as reflected by PGE_2 levels.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** The following chemicals and reagents were purchased from the suppliers listed: curcumin, collagenase, uridine 5'-diphosphateglucuronol acid, uridine 5'-diphospho-N-acetyl glucosamine, magnesium chloride, uridine 5'-diphosphoglucuronosyl transferase, bacterial β-glucuronidase (type VII-A from Escherichia coli), sulfatase (type VIII from Abalone entails), EGTA, glycerolformal, 1,4-dioxane (anhydrous), heparin, sodium borohydride, PMA, human serum albumin, and arachidonic acid (Sigma-Aldrich Comp. Ltd., Poole, Dorset, United Kingdom); HPLC-grade acetonitrile (Fisher Laboratory Supply Ltd., Loughborough, United Kingdom); HBSS (Hanks' balanced salt solution; Gibco, Paisley, United Kingdom); pentobarbionate (Sagatal; Rhone Merieux Ltd., Harlow, Essex, United Kingdom); sulfur trioxide-N-triethylamine complex (Fluka Chemicals, Gillingham, Dorset, United Kingdom); DMEM, ammonium acetate (Merck Ltd., Poole, Dorset, United Kingdom); halothane (Zenea, Macclesfield, Cheshire, United Kingdom); Bacto Soltran kidney perfusion solution (Baxter Healthcare, Berkshire, United Kingdom); PGE_2 immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Tetrahydrocurcumin and hexahydrocurcumin were synthesized as described (17) and provided by Dr. W. Wang (Phytopharm plc, Cambridge, United Kingdom).

**Cells and Animals.** Nonmalignant HCECs (18) were obtained from Dr. A. Pfeifer (Nestlé Research Institute, Lausanne, Switzerland). These cells were passaged in B5 medium (Biofluids Inc., Rockville, MD) containing BSA, bovine pituitary extracts, retinoic acid, vitamin C, and deoxymethasone. Male (180-200 g) or female (160-180 g) F344 rats were purchased from Charles River UK Ltd. (Margate, Kent, United Kingdom) or Harlan UK Ltd. (Bicester, Oxon, United Kingdom). Rats that were maintained in a purpose-built animal house in negative pressure isolators (19-23°C) under a 12-h light/dark cycle received RM1 rodent maintenance diet (SDS, Kent, United Kingdom) and water ad libitum. Experiments using animals were conducted as stipulated by Project License 80/1250 granted to the Medical Research Council Toxicology Unit by the United Kingdom Home Office, and the experimental design was vetted and approved by the Leicester University Ethical Committee for Animal Experimentation.

**Isolation of Human and Rat Hepatocytes.** Isolation of hepatocytes from humans or rats was performed by the collagenase perfusion method of Berry and Friend (19) according to the protocol described by Seglen (20). Healthy liver tissue resected from four Caucasian patients with secondary hepatic tumors (two females, 38 and 61 years old; two males, 51 and 53 years old) were obtained from the United Kingdom Human Tissue Bank (Leicester, United Kingdom). Patients had not received medication known to interfere with liver metabolic activity. Following removal of the liver from the body, cannulas were immediately inserted into four to five large blood vessels of the lobe, which was immediately perfused in theater with kidney perfusion medium (500 ml) and transported in this fluid on ice. On arrival in the laboratory, the liver was transferred to a custom-built stainless steel tank and perfused for 20-30 min with liver perfusion medium maintained at 37°C to remove blood. The liver was then perfused with liver digestion media for approximately 45 min. The digested liver lobe was transferred to a tray containing liver suspension medium (DMEM supplemented with human serum albumin 2%), and the tissue was gently disrupted to release cells. Under digested tissue was removed by passing the cell suspension through a series of sieves (successive mesh size: 1 mm, 0.5 mm, and 100 μm) for the isolation of rat liver cells, male F344 rats (180-220 g) were anesthetized with pentobarbionate, and the liver was perfused (5 min; rate, 50 ml/min) via the inferior portal vein with HBSS (containing 1 mm EGTA), which had been presaturated with carbogen (oxygen/CO₂ 5%). The liver was digested using collagenase (100 mg/liter) and calcium chloride (332 mg/liter) in HBSS. Tissue was gently disrupted and washed through a sieve (100-μm mesh size) with liver suspension medium. Human or rat cells, thus, obtained were washed three times and centrifuged (3 min, 30 X g, 4°C). Cells were counted using a hemocytometer immediately following isolation. Hepatocyte viability determined by the trypan blue exclusion assay was routinely 80% or above. Hepatocytes in suspension were maintained on ice for a maximum of 30 min before use.

**Incubations with Hepatocytes.** Freshly isolated hepatocytes (2×10⁶ cells per ml) were suspended in liver suspension medium (2 ml) and incubated in a slowly shaking incubator (37°C). Curcumin dissolved in DMSO was added to furnish a final concentration of 100 μM. The concentration of DMSO (maximally 0.1% v/v) in the incubate did not interfere with cell viability. Control incubates included curcumin with heat-inactivated hepatocytes or hepatocytes incubated with the vehicle only. Incubations were terminated after 5, 30, 60, and 120 min by placing vials on dry ice. During the longest incubation period (2 h) cell viability decreased to between 60% and 40% of initial values (trypan blue exclusion test). Before HPLC analysis, suspensions were rapidly defrosted, immediately extracted twice with ethyl acetate (twice volume of sample), and mixtures were centrifuged (2800 X g, 4°C, 15 min). The organic layers were removed, combined, and evaporated to dryness under nitrogen. Samples were reconstituted in acetonitrile and immediately analyzed by HPLC. In control experiments, the ability of hepatocytes to conjugate the model substrate umbelliferyl was assessed and found to be intact (21).

**Metabolism Studies in Vivo.** Female F344 rats received curcumin either p.o. (gavage, 500 mg/kg; vehicle, DMSO; dosage volume, 2.0 ml/kg) or i.v. (40 mg/kg; vehicle, glycerol formal; dosage volume, 1.0 ml/kg). The p.o. dose level was chosen because it is approximately equivalent to a daily dose of curcumin when ingested with the diet at 1%, a concentration that has been frequently used in intervention studies. The i.v. dose chosen was the highest feasible dose formulated in a solvent suitable for injection. Animals were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture 30 min and 1, 2, 6, 12, and 24 h (p.o. administration) or 5 and 30 min and 1 and 6 h (i.v. administration) after dosing. Blood was also obtained from animals that had received vehicle only. Blood was transferred to heparinized centrifuge tubes, and plasma was obtained by centrifugation (1100 X g, 4°C, 25 min). Aliquots of plasma were extracted with twice the volume of ethyl acetate, or mixed with four times the volume of a mixture of DMSO:methanol (1:4). The mixtures were centrifuged (1000 X g, 15 min), and the supernatant was removed. In the case of the ethyl acetate extract, the organic layer was evaporated under nitrogen. Extraction efficiencies from plasma using the ethyl acetate extraction method for curcumin, hexahydrocurcumin, and curcumin sulfate were determined by HPLC (see below) as 95 ± 4%, 70 ± 5%, and 49 ± 9%, respectively. Recovery of curcumin in the case of treatment with DMSO:methanol was 70 ± 5%
the quantity of curcumin and its putative metabolites that is similar but not identical to that described before (13). A Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410) and a Symmetry Shield RP 18 column (150 x 3.9 mm; Waters) were used. Detection of curcumin, curcumin sulfate, and curcumin glucuronide was achieved at 420 nm, whereas tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol were analyzed at 280 nm. Tetra-(m-hydrophenyl)-chlorin was used as an internal standard. Samples were reconstituted in acetonitrile:water (1:1), and the injection volume was 50 μl. A linear gradient of 5–45% acetonitrile in 0.01% ammonium acetate (pH 4.5) was used for 30 min, followed by an increase over 20 min to 95% acetonitrile (flow rate, 1 ml/min). The retention times quoted in the results and in Table 1 were obtained using these conditions. The limits of detection of curcumin, tetrahydrocurcumin, and hexahydrocurcumin in plasma and hepatocyte suspensions were between 5 and 10 nm. In the case of curcumin, the quantitative method was validated using a 2.7-μM solution yielding intra- and interday coefficients of variation of 5.1% and 9.8%, respectively (n = 4), and a limit of quantitation of 20 nm. Curcumin calibration curves spanned the concentration range of 20 pm to 40 μM.

Synthesis of Curcumin Sulfate and Curcumin Glucuronide. For the synthesis of curcumin sulfate, curcumin (1.36 mmol), dissolved in anhydrous 1,4-dioxane, was incubated with sulfur trifluoride N-triethylamine complex (6.8 mmol) and maintained at 37°C for 2 h. The precipitate was washed (ethyl acetate) to remove unreacted curcumin. For the synthesis of curcumin glucuronide according to a published method (22), curcumin (1 mm), uridine diphospho-N-acetyl glucosamine (2 mM), HEPES buffer (25 mM, pH 7.4), magnesium chloride (10 mM), and uridine diphosphoglucuronosyl transferase (150 units/liter) were incubated (3 ml, 37°C, 3 h). The incubation medium was extracted twice with ethyl acetate, and the combined organic extracts were evaporated under nitrogen. The residues of either reaction were reconstituted in acetonitrile:water (1:1). Eluent corresponding to the peaks that were tentatively assigned to curcumin sulfate or curcumin glucuronide was collected, and the solvent removed from the collected fractions by freeze-drying. The isolated materials were reanalyzed by HPLC (detection at 420 nm). The extinction coefficients of curcumin sulfate and curcumin glucuronide were approximately equivalent to that of curcumin, as established by a standard curve (see below). Consequently, their quantitation in the plasma was based on calibration curves established with curcumin. The structural identity of the products as curcumin sulfate and curcumin glucuronide was confirmed by mass spectrometry (see "Results" and Table 1).

Synthesis of Hexahydrocurcuminol. An equimolar amount of sodium borohydride was added to hexahydrocurcumin (3 mM) dissolved in methanol. HPLC analysis (detection at 280 nm) showed that after 2 h at ambient temperature all of the hexahydrocurcumin had reacted. Methanol was removed by evaporation under nitrogen, and the residue was reconstituted in water (2 ml) and adjusted to pH 4.5. The product was extracted with ethyl acetate, and the solvent was evaporated under nitrogen. The structural identity of the product as hexahydrocurcuminol was confirmed by mass spectrometry (see "Results" and Table 1).

Table 1 Characterization of major curcumin metabolites by HPLC and mass spectrometry

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC retention time (min)</th>
<th>Mass spectrometric product ions (m/z)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexahydrocurcumin</td>
<td>22.5</td>
<td>135 (100); 375 (50*); 199 (32); 179 (22)</td>
</tr>
<tr>
<td>Hexahydrocurcuminol</td>
<td>25</td>
<td>177 (100); 369 (72); 545 (15)*</td>
</tr>
<tr>
<td>Curcumin sulfate</td>
<td>31</td>
<td>367 (100); 217 (85); 149 (61); 447 (50)*</td>
</tr>
<tr>
<td>Hepatocyte metabolite 1</td>
<td>24.4</td>
<td>179 (100); 165 (18); 193 (17); 373 (10)</td>
</tr>
<tr>
<td>Hepatocyte metabolite 2</td>
<td>22.5</td>
<td>135 (100); 375 (67); 179 (23); 199 (22)</td>
</tr>
<tr>
<td>Plasma metabolite 1</td>
<td>25</td>
<td>545 (100); 369 (45); 177 (30)</td>
</tr>
<tr>
<td>Plasma metabolite 2</td>
<td>31</td>
<td>447 (100); 367 (25); 217 (24); 149 (22)</td>
</tr>
</tbody>
</table>

* m/z values of the most prominent product ions in the mass spectrum; the percentage abundance is in parentheses. For details of chromatography and spectrometry see "Materials and Methods."
peak that coeluted with curcumin (data not shown). Although 35% of the initial amount of curcumin was still present in suspensions of human hepatocytes after incubation for 2 h, curcumin concentrations were reduced to near the detection limit when incubated with rat hepatocytes for that time period. In addition to curcumin, there were two small peaks in extracts from both types of hepatocytes, characterized by retention times of ~25 and 31 min, consistent with curcumin sulfate and glucuronide, respectively (see below). HPLC analysis using detection at 280 nm yielded at least four metabolite peaks; the two major ones were characterized by retention times of 22.5 and 24.4 min (Fig. 2). Both species were found in hepatocytes from humans (Fig. 2A) and rats (Fig. 2B), and they were absent from chromatograms of hepatocyte incubations from which curcumin had been omitted. The peak characterized by the retention time of 24.4 min coeluted with authentic hexahydrocurcumin, and mass spectrometric analysis of a dried residue of the eluent was collected at the pertinent retention time confirmed its identity (Table 1). The other major metabolite with a retention time of 22.5 min afforded a molecular ion of m/z 375 on mass spectrometric analysis (Table 1), thus containing two mass units more than hexahydrocurcumin. In a separate experiment, authentic hexahydrocurcumin was incubated with rat hepatocytes and rapidly metabolized to the species characterized by a retention time of 22.5 min and the molecular ion m/z 375. These findings are consistent with the possibility that the metabolite was generated from curcumin via hexahydrocurcumin. Furthermore, the same molecule was generated chemically on treatment of hexahydrocurcumin with the reducing agent sodium borohydride. We infer from these results that the second major hepatocytic metabolite of curcumin is hexahydrocurcuminol (Fig. 1). Fig. 3 shows the time course of disappearance of curcumin and concurrent generation of its two major metabolites in suspensions of rat hepatocytes. The ratio of integrated peak areas of hexahydrocurcumin over hexahydrocurcuminol after incubation of rat or human hepatocytes with curcumin for 2 h furnished values of 1.0 ± 0.1 in the case of rat hepatocytes as compared with 3.2 ± 0.6 (mean ± SD, n = 3 for each) for human hepatocytes. Taken together, the results obtained in experiments with hepatocytes demonstrate, first, that metabolic reduction of curcumin to hexahydrocurcumin is rapid, followed by the reduction of the carbonyl moiety to hexahydrocurcuminol, and, second, that overall reduction of curcumin to hexahydrocurcuminol, the ultimate reduction product of curcumin, occurs more extensively in rat than in human hepatocytes.

**Metabolism of Curcumin in Vivo.** Metabolites were characterized in rat plasma in vivo after administration of curcumin via the i.v. (40 mg/kg) or p.o. (0.5 g/kg) routes. Plasma samples were analyzed by HPLC with detection either by UV absorption at 420 nm or by ion-selected monitoring in mass spectrometry mode. Spectrophotometric analysis of plasma at 280 nm did not allow useful inferences to be made because specific curcumin metabolite peaks were indistinguishable from a host of peaks attributable to endogenous constituents. Plasma from rats that had received curcumin p.o. afforded a peak that coeluted with curcumin, but at levels below the limit of quantitation (data not shown). In addition, there were three metabolites harboring the intact diferoylmethane structure with retention times of approximately 20, 25, and 31 min in the plasma of rats after both routes of administration. Incubation of plasma extracts with β-glucuronidase led to a reduction of the height of the peak with a retention time of 25 min with a concurrent increase in the peak height of parent curcumin (data not shown). Similarly, incubation of the plasma extracts

![Fig. 3. Time course of disappearance of curcumin (A) and generation of metabolites hexahydrocurcumin (B) and hexahydrocurcuminol (C) in suspensions of rat hepatocytes. HPLC detection was by UV at 280 nm. The results are the mean ± SD of three incubations with separate hepatocyte preparations. For details of hepatocyte isolation, incubation, and HPLC analysis see "Materials and Methods."](image-url)

![Fig. 4. Mass spectrometric analysis by selected ion monitoring at the indicated m/z values of extracts of rat plasma 30 min after administration of curcumin (40 mg/kg, i.v.). Peaks can be assigned to curcumin (1; m/z = 367), hexahydrocurcumin (2; m/z = 373), hexahydrocurcuminol (3; m/z = 375), and hexahydrocurcumin glucuronide (4; m/z = 549). Note that retention times are longer than those shown in Fig. 2 and Table 1 because the chromatographic conditions were slightly different; the retention times shown here are 67.1 min for curcumin, 39.5 min for hexahydrocurcumin, 38 min for hexahydrocurcuminol, and 24.9 min for hexahydrocurcumin glucuronide. Selected ion chromatograms of plasma extracts from rats that had not received curcumin did not show any of the peaks seen here. The chromatograms are representative of three experiments. For details of curcumin administration and HPLC and mass spectral analyses see "Materials and Methods."](image-url)
The identity of the cumin, which furnished the two small peaks just beyond curcumin glucuronide and curcumin sulfate (2), and curcumin glucuronide (1; see "Results"). Note that commercially available curcumin contains 15% desmethoxycurcumin and 5% bisdesmethoxycurcumin, which furnished the two small peaks just beyond curcumin. The arrow in Fig. 5A marks the position of a peak characterized by mass spectrometry as curcumin glucuronide sulfate (see "Results"). HPLC analysis of extracts of plasma from control rats did not furnish any detectable peaks. All absorbance units. The chromatogram is representative of three experiments, and the values in B are the mean ± SD values of three separate animals. For details of curcumin administration and HPLC analysis see "Materials and Methods.

The results outlined above allow the following four novel conclusions to be drawn concerning the metabolism of curcumin, which contribute to the understanding of its preclinical pharmacology and, thus, aid with the planning of its clinical evaluation: (a) human and rat liver reduces curcumin first to hexahydrocurcumin and then to hexahydrocurcuminol, whereas conjugation of curcumin is only a minor hepatic biotransformation route; (b) the biotransformation step curcumin → hexahydrocurcumin is rapid, and the overall rate of curcumin reduction seems slower in human than in rat liver cells; (c) the predominant metabolites of curcumin in rat plasma in vivo are curcumin glucuronide and curcumin sulfate, whereas hexahydrocurcumin and hexahydrocurcuminol, the major metabolites of curcumin in hepatocyte suspensions, occur only in small amounts in rat plasma after curcumin administration; (d) curcumin metabolites are markedly less able to inhibit inducible COX-2 expression than their metabolic progenitor.

Whereas tetrahydrocurcumin, hexahydrocurcumin, and curcumin glucuronide have been described as products of the metabolic reduc-
tion of curcumin in rodents before (13, 15), this is the first study that describes hexahydrocurcuminol and curcumin sulfate as curcumin metabolites. Hexahydrocurcuminocin occurs naturally in the rhizomes of the ginger plant Zingiber officinale (24) and of Curcuma xanthorrhiza (17), the major plant source of curcumin. Our results suggest that curcumin glucuronide and curcumin sulfate are generated only in small amounts in hepatocytes, whereas they are abundant in rat plasma after administration of curcumin. This discrepancy is consistent with the hypothesis that they are generated, at least in part, extra-hepatically, probably in the gastrointestinal tract (25). The metabolic conversions described here and their interrelationships are described in Fig. 7: the figure shows that curcumin undergoes metabolism to its sulfate and glucuronide and sulfate-glucuronide conjugates. The liver reduces curcumin to hexahydrocurcumin and hexahydrocurcuminol, probably via the intermediacy of dihydrocurcumin and tetrahydrocurcumin, two species that were identified in mice (13), but not in the present study in rat plasma or rat and human hepatocytes. Dihydrocurcumin, tetrahydrocurcumin, and hexahydrocurcumin generate glucuronides, all three of which were characterized in mice (13), and hexahydrocurcumin glucuronide was also found here in rats. Hexahydrocurcuminol constitutes the ultimate product of curcumin reduction, and it is conceivable that it is also a substrate of conjugating enzymes. However, identification of hexahydrocurcuminol glucuronide or hexahydrocurcuminol sulfate has thus far been elusive.

Curcumin and its metabolites have not been compared before in terms of pharmacological potency. Inhibition of prostaglandin biosynthesis by inhibition of COX-2 induction is arguably an important mechanism that contributes to the chemopreventive activity of curcumin (11). Here, we show that stepwise metabolic reduction of the diarylheptanoid one chain was accompanied by a significant loss of ability to inhibit COX-2 expression in a bioassay as reflected by PGE\textsubscript{2} activity. Of the reduced species, tetrahydrocurcumin, found as glucuronide in the plasma of mice (13) but not in human or rat hepatocytes, and hexahydrocurcumin, a major curcumin metabolite in human and rat hepatocytes, were less capable than curcumin of interfering with the induction of PGE\textsubscript{2} production and COX-2 expression. Hydroxycurcuminocin, the other major metabolite of curcumin in the liver, lacked COX-2-suppressing activity, and curcumin sulfate interfered only weakly with COX-2 expression. It is pertinent to mention that the data presented above does not allow judgement as to whether the effect of curcumin and its metabolites on COX-2 expression is concentration dependent. The results render it unlikely that the major hepatic metabolites of curcumin are responsible for, or contribute in a major way to, its chemopreventive activity via the inhibition of COX-2 expression. It is, therefore, possible that the metabolic conversions of curcumin described here and shown in Fig. 7 are pharmacological deactivation pathways. Information as to the biological potency of curcumin metabolites is scarce. Only tetrahydrocurcumin has previously been subjected to comparative pharmacological studies. It was found to be more potent than curcumin in the carrageenan-induced rat paw edema test for anti-inflammatory activity (26), and at least as potent an antioxidant as curcumin in rabbit erythrocyte membrane ghosts and rat liver microsomes in vitro (27, 28). In contrast, tetrahydrocurcumin was much less potent than curcumin as inducer of quinone reductase in cells in vitro (29) or as inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin (30). Our results intimate that the unsaturated nature of the diarylheptanoid chain and free phenolic moieties may be pivotal pharmacopoeic features of molecules related to curcumin for optimal inhibition of COX-2 expression, the in vitro paradigm of chemopreventive activity chosen here.

The prolonged presence in rat plasma of curcumin glucuronide and curcumin sulfate after oral administration as described here may be the corollary of slow absorption of curcumin from the gastrointestinal tract and/or of its intrahepatic circulation. This contention is consistent with results of a recent drug distribution study in mice (13). It suggests low, probably subefficacious, curcumin levels in a variety of tissues, which amounted to between 1 nmol/g in brain and 72 nmol/g in liver 1 h after an i.p. dose of 100 mg/kg of the drug. The intestine was the exception in that it contained 300 nmol/g tissue. The results presented here, together with information published previously, suggest that curcumin taken p.o. might prevent cancer of the colon more effectively than malignancies in other tissues. This conclusion provides a rationale for trials of curcumin to be conducted with the aim of preventing human colorectal cancer.

In conclusion, the results described here shed new light on the role of the liver in the metabolic fate of curcumin because they suggest that hepatic metabolism of curcumin is a pharmacological deactivation step. Overall, the results buttress the rationale for clinical evaluation of curcumin in the chemoprevention of human colorectal cancer. The relevance of the findings discussed here for humans who consume curcumin will eventually be established in clinical studies of curcumin, in which pharmacokinetic and pharmacodynamic parameters will be correlated.

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