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Cancer Chemopreventive Agent Curcumin metabolism 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. Curcumin sulfate was identified in incubations of curcumin with intact rat gut sacs. Curcumin was sulfated by human phenol sulfotransferase 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 are more metabolisms 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.

Keywords: Cancer, Chemopreventive Agent, Curcumin Metabolism.

Contents

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1. 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 subcon1tinent 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, antineoplastic, and anti-inflammatory properties[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, 6, 7, 8, 9] , and it interferes with lipid peroxidation [10, 11, 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]. 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 [^{3}H] labeled curcumin was incubated with inverted rat gut sacs, and biotransformation was deduced from the disappearance of radioactivity associated with the parent compound [24].

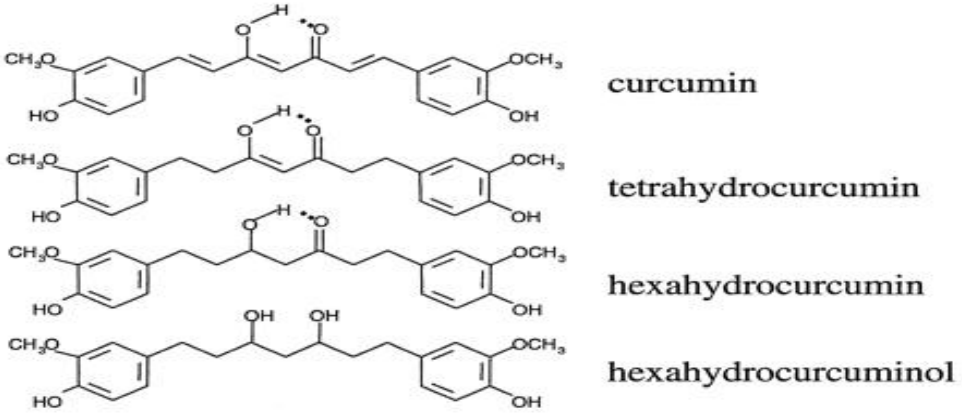

Figure 1. Chemical structure of curcumin and products of its bio reduction

2. Materials and Method

Chemicals and Reagents:

The following chemicals and reagents were purchased from the suppliers listed: curcumin, uridine 5′-diphosphoglucuronic acid, magnesium chloride, PAPS,2 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]. 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.

Table1. Metabolites of curcumin in cytosolic and microsomal fractions of liver and intestine of humans and rats.

Metabolites	**Intestine (nmol/mg protein)**						**Liver (nmol/mg protein)**		
		Cytosol		**Microsomes**			**Cytosol**		
		Rat	**Human**	**Rat**	**Human**	**Rat**	**Human**	**Rat**	**human**
Hexahydrocurcumin	7+-2	140+-90	N.D	N.D	24+-11	120+-114	N.D	N.D	
Curcumin sulfate	24+-2	102+-22	N.D	N.D	180+-38	38+-7	N.D	N.D	
Curcumin glucuronide	N.D	N.D	15+-1	254+-228	N.D	N.D	978+-148	93+-28	

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. 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). 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... 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 Using 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 carbon 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 m, pH 8.4 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 acetate 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). 5,10,15,20-Tetra-chlorine served as internal standard. Detection of curcumin, curcumin sulfate, and curcumin glucuronide was achieved at 420 nm, whereas tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol were analyzed at 280 nm. 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 quadruple mass spectrometer upgraded to Quattro MK II specifications 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] .

3. Results and Discussion

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. 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 hexahydrocurcuminol (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).

Fig.2. 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 with detection at 280 (A and D) and 420 nm (B, C, E, and F). The identity of the peaks was established by cochromatography and mass spectrometry as curcumin (peak 3), hexahydrocurcumin [1], tetrahydrocurcumin [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-hydroxylphenyl)-chlorine. Note that commercially available curcumin contains 15% desmethoxycurcumin and 5% bisdesmethoxycurcumin, which furnished two small peaks just beyond curcumin (see especially in A and D)

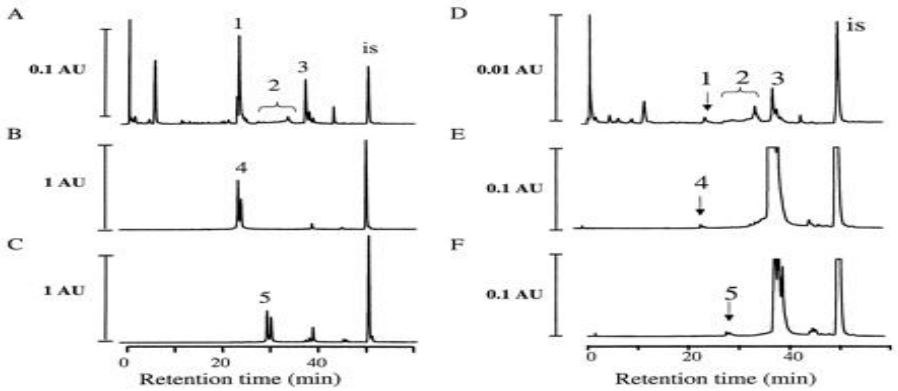

Figure 2. On-line HPLC-mass spectrometry analysis in selected ion registration mode of curcumin sulfate

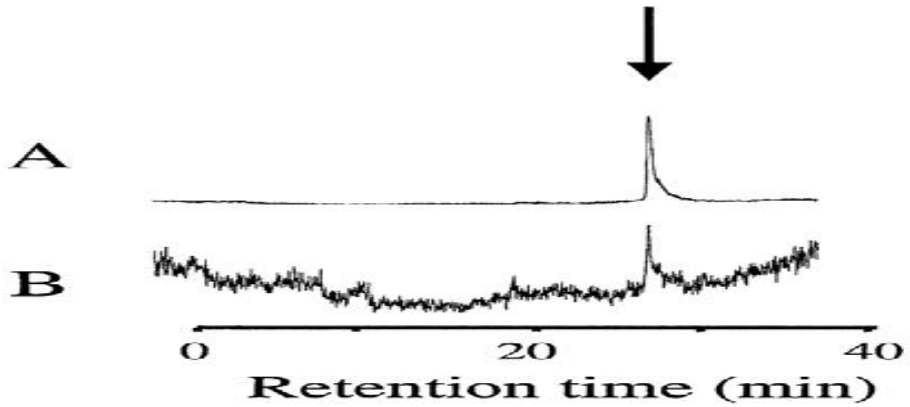

Figure.3

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). 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 proteins, 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).

Metabolism of Curcumin by Intact Rat Gut:

To explore the metabolism of curcumin in an intact intestinal tissue, the agent was incubated with inverted rat gut sacs. Curcumin sulfate was unequivocally identified by cochromatography and mass spectrometry (result not shown). Curcumin glucuronide and products of metabolic curcumin reduction were not detected.

Nature and Location of Curcumin SULTs and Reeducates:

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 and SULT1A3 metabolize curcumin to its 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. Western blot analyses of human intestinal and hepatic tissue samples with monoclonal antibodies against phenoltransferase isoenzymes SULT1A1 and 1A3. Assignation of lanes is as follows: A, recombinant SULT1A3; F, recombinant SULT1A1; B, C, and D, samples of intestine from 3 humans; E, sample of liver from 1 human. Arrows, positions of the SULT1A3 (solid arrow) and SULT1A1 (broken arrow) bands; in B–E, the top bands are indistinguishable from that of SULT1A3, and the bottom bands comigrate with SULT1A1. For details of Western analysis, see "Materials and Methods." The blots are representative of two separate experiments.

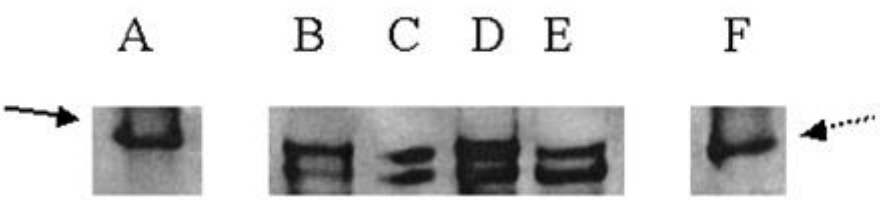

Figure.4

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. Both hexahydrocurcumin and hexahydrocurcuminol have been reported to be the predominant products of metabolic reduction of curcumin in incubations of intact human or rat hepatocytes [18] , In contrast, human gut cytosol reduced curcumin easily (see Fig. 2), and it also reduced hexahydrocurcumin to hexahydrocurcuminol. Fig.5. High-performance liquid chromatograms of extracts of incubations of hexahydrocurcumin (A) or curcumin (B) with human intestinal microsomes. Incubation time was 90 min, and analysis was conducted with detection at 280 nm. Peak allocation is hexahydrocurcumin (peak 1), hexahydrocurcuminol (6), and curcumin (3).

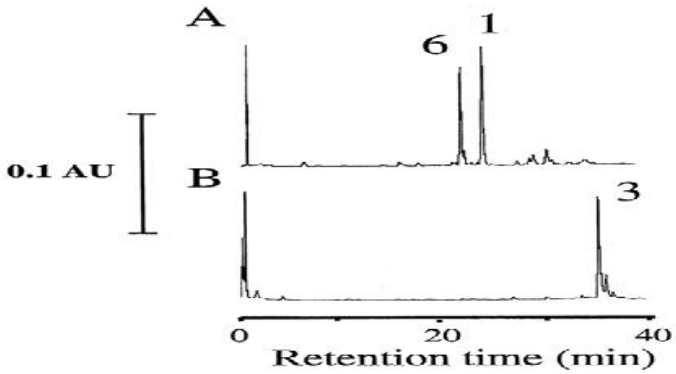

Figure.5

These are the first results to provide convincing evidence that curcumin is bio transformed 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. . 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..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 extra hepatic 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 oxido-reductases reduces curcumin and thus contributes to the formation of curcumin reduction products in vivo.

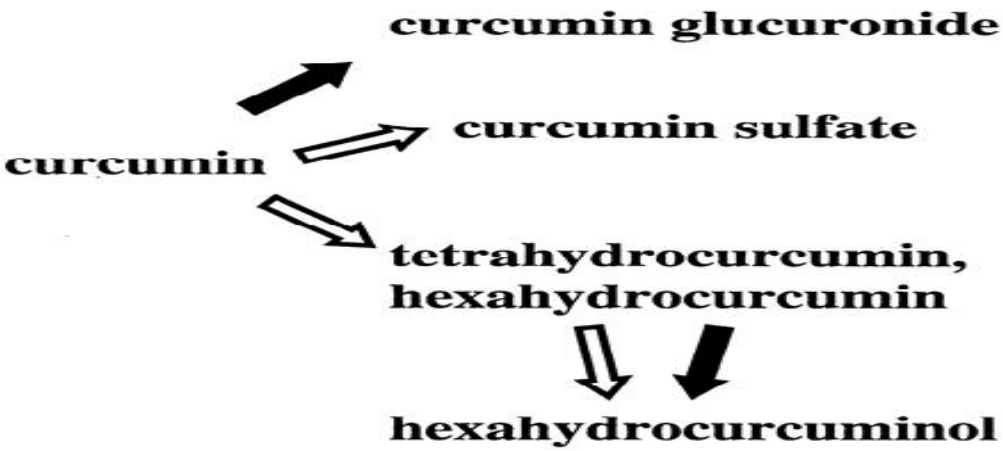

Figure 6. Schematic representation of metabolism of curcumin in intestinal and hepatic cytosol (open arrows) and microsomes (closed arrows).

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 pharmaceutical profile, exemplified by extremely low aqueous solubility.

4. Conclusion

Tetrahydrocurcumin was found to be more potent than curcumin in the carrageenan-induced rat paw edema test for anti-inflammatory activity and at least as potent an antioxidant as curcumin in rabbit erythrocyte membrane ghosts and rat liver microsomes in vitro In contrast, tetrahydrocurcumin was much less potent than curcumin as and inducer of quinone reductase in cells in vitro or as an inhibitor of phorbol 12-myristate 13-acetate-induced tumor promotion in mouse skin. Furthermore, in a comparative analysis of the ability of five curcumin metabolites, curcumin sulfate, curcumin glucuronide, tetrahydrocurcumin, hexahydrocurcumin, and hexahydro- curcuminol, 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. On balance, these findings justify the tentative conclusion that the intestinal biotransformation of curcumin constitutes a pharmacological deactivation process, in that metabolism generates 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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