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Metabolism of Curcuminoids in Tissue Slices and Subcellular Fractions from Rat Liver

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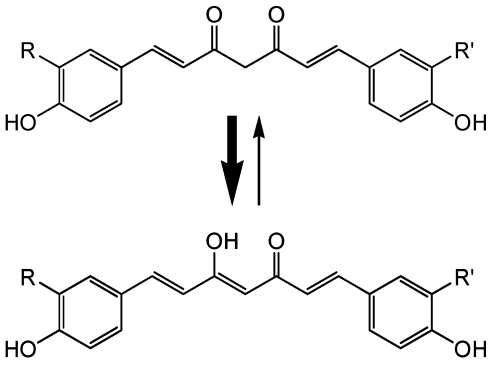
Curcumin and its natural congeners are of current interest because of their putative anti-inflammatory and anticarcinogenic activities, but knowledge about their metabolic fate is scant. In the present study conducted with precision-cut liver slices from male and female Sprague−Dawley rats, five reductive but no oxidative metabolites of curcumin and its demethoxy and bis-demethoxy analogues were observed and identified by HPLC and GC-MS analysis, mostly by comparison with authentic reference compounds. The major reductive metabolites were the hexahydrocurcuminoids in both male and female rat liver slices, whereas male rats formed more octahydro than tetrahydro metabolites and female rats more tetrahydro- than octahydrocurcuminoids. Tetrahydro, hexahydro, and octahydro metabolites were predominantly present as glucuronides, but a significant proportion of sulfate conjugates was also observed. The lack of formation of oxidative metabolites of curcumin and the ready generation of reductive metabolites were confirmed using rat liver microsomes and cytosol, respectively. Results of enzymatic hydrolysis studies conducted under various conditions revealed that curcumin and demethoxycurcumin are chemically less stable than bis-demethoxycurcumin, whereas the reductive metabolites of all three curcuminoids are stable compounds. This is the first report on the metabolism of demethoxycurcumin and bis-demethoxycurcumin. In view of the chemical instability of the parent curcuminoids, it is proposed to use their major phase I metabolites, that is, the stable hexahydro products, as biomarkers for exposure in clinical studies.

KEYWORDS: Curcumin; liver slices; curcuminoids; reductive metabolites; instability

INTRODUCTION

Curcuminoids are natural yellow-orange pigments present in the rhizomes of the Asian plant *Curcuma longa* and other *Curcuma* species, for example, *C. xanthorriza*. They are commonly isolated from the spice and food-coloring agent turmeric. *Curcuma* extracts contain three diarylheptanoids, namely, curcumin as the major component together with smaller amounts of demethoxycurcumin and bis-demethoxycurcumin (*1*). As depicted in **Figure 1**, curcuminoids have a unique conjugated structure including a β-diketone moiety due to which they undergo keto−enol tautomerization and exist entirely in the enol form in solution (*2*, *3*).

In traditional Oriental medicine, turmeric has been used as a medicinal herb for thousands of years. Curcumin is recognized for its broad spectrum of biological activities including cholagogic and choleretic, anti-inflammatory, and antioxidant activities, the most important being its putative anticarcinogenic effect (*4*−*6*). Curcumin acts as a scavenger of reactive oxygen species and interferes with lipid peroxidation (*7*, *8*). The antioxidant



	R	R'
Curcumin	OCH_3	OCH_3
Demethoxy-curcumin	H	OCH_3
Bis-demethoxy-curcumin	H	H

Figure 1. Chemical structures of the curcuminoids from turmeric.

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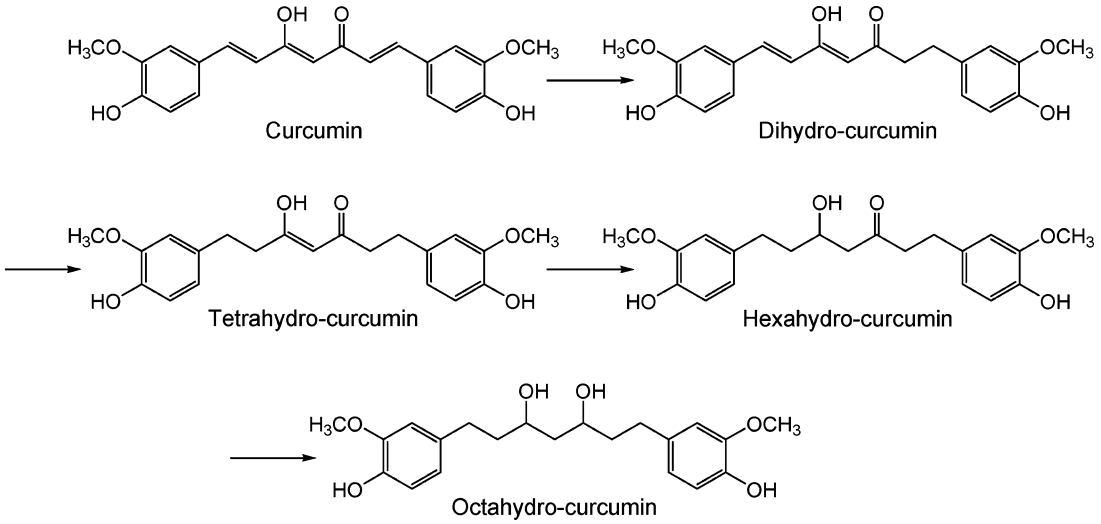

Figure 2. Reductive metabolism of curcumin.

properties of curcumin are assumed to be imparted by its polyphenolic structure, the β-diketone moiety, and the formation of relatively stable free radicals due to its extended conjugated double-bond system (*5*, *9*). The anti-inflammatory and cancer-preventive properties have been linked to numerous biological activities including effects on arachidonate metabolism through inhibition of both cyclooxygenase and lipoxygenase pathways (*5*, *10*, *11*). Curcumin is also a potent inhibitor of mutagenesis and chemically induced carcinogenesis (*5*, *12*−*14*).

Although numerous aspects of the pharmacology of curcumin, in particular its activity as a chemopreventive agent, have been studied, the absorption, pharmacokinetics, and metabolism in humans and experimental animals have not been fully characterized. The metabolism of curcumin, both phase I and phase II, has mostly been studied in rats in vivo and in vitro (*15*−*21*). More recently, information on the metabolism of curcumin in humans has been obtained from in vitro studies with hepatic and intestinal cells and subcellular fractions (*20*, *21*), as well as from clinical studies with cancer patients (*22*−*25*). The present knowledge can be summarized as follows: phase I metabolism comprises the successive reduction of the four double bonds of the heptadiene-3,5-dione system (**Figure 2**). Tetrahydrocurcumin and hexahydrocurcumin are the major products observed in most studies, whereas dihydrocurcumin and octahydrocurcumin (also named hexahydrocurcuminol) usually represent minor products or are not detected at all. The enzymes responsible for the bioreduction have been found to reside in the cytosol of liver and intestine and include alcohol dehydrogenase. Curcumin and its reduced metabolites appear to be easily conjugated in vivo and in vitro. Reported conjugates of curcumin include a monoglucuronide, a monosulfate, and a mixed sulfate/glucuronide. Although mass spectra of these phase II metabolites have been published, their exact chemical structures have not yet been established. Glucuronidation and sulfation of curcumin are believed to occur in the liver and intestine of rats and humans. No data have yet been published on the metabolism of demethoxycurcumin and bis-demethoxycurcumin.

The aim of the present study was to compare the phase I and phase II metabolism of curcumin, demethoxycurcumin, and bis-demethoxycurcumin in precision-cut tissue slices prepared from the liver of male and female rats. Of particular interest was the question of whether oxidative phase I metabolites would be formed, for example, through hydroxylation or demethylation of the curcuminoids. Liver slices represent a valuable model for biotransformation studies because the physiological liver microarchitecture, that is, cell-to-cell contacts as well as cell−matrix interactions, is maintained. Thus, the in vivo situation concerning phase I and phase II metabolism is reflected very well in tissue slices (*26*, *27*).

MATERIALS AND METHODS

Chemicals and Animals. Curcumin, demethoxycurcumin, bis-demethoxycurcumin, and tetrahydrocurcumin were provided by the Arizona Center for Phytomedicine Research (Tucson, AZ): tetrahydrocurcumin had been chemically synthesized by Sabinsa Corp. (Payson, UT), whereas the other three curcuminoids were isolated from turmeric and purified by column chromatography and recrystallization. All four compounds had a purity of >99% according to HPLC analysis. Their HPLC retention times and UV−vis spectra are given in **Table 1**. Pure curcumin and bis-demethoxycurcumin were also chemically synthesized as described below. Commercial curcumin obtained from turmeric and containing 75% curcumin, 18% demethoxycurcumin, and 7% bis-demethoxycurcumin, sulfatase (type VI from *Aerobacter aerogenes*), β-glucuronidase (type B-1 from bovine liver), alcohol dehydrogenase (from equine liver), and all other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (Steinheim, Germany). β-Glucuronidase/arylsulfatase from *Helix pomatia* was purchased from Roche Diagnostic Co. (Mannheim, Germany). HPLC grade acetonitrile was from Carl Roth Co. (Karlsruhe, Germany).

Male and female Sprague−Dawley rats were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Animals were kept under a 12-h light/dark cycle and received water and commercial laboratory chow ad libitum. Rats of 200−300 g weight were used for the preparation of liver slices and hepatic microsomes and cytosol.

Chemical Syntheses. Curcumin and bis-demethoxycurcumin were synthesized according to the method of Pabon et al. (*28*). Briefly, 2.5 mL of acetylacetone (25 mmol) and 1.25 g of boric acid anhydride (17 mmol) were stirred for 0.5 h to form a thick paste, to which 10 mL of dry ethyl acetate was added. This mixture was then added to a solution of 50 mmol of the respective substituted benzaldehyde (7.6 g of vanillin for curcumin, 6.1 g of 4-hydroxybenzaldehyde for bis-demethoxycurcumin) and 26.2 mL of tributylborate (100 mmol) in 15 mL of dry ethyl acetate. After 10 min of stirring, 0.5 mL of *n*-butylamine (5 mmol) was added dropwise over a period of 10 min, and stirring was continued for 4 h. The mixture was then kept without stirring overnight, followed by the addition of 37.5 mL of 0.6 N aqueous HCl at 60 °C and stirring for 1 h to hydrolyze the reaction product. The organic layer was separated and the aqueous layer extracted three times with ethyl acetate. The combined organic phases were washed acid-free and dried over Na_2SO_4, and solvent was evaporated under reduced pressure. The synthetic curcuminoids were recrystallized from methanol at 4 °C and washed several times with cold methanol and

Table 1. Chromatographic and Spectrometric Properties of Curcuminoids and Their Reductive Metabolites.

compound	HPLC (min)	UV–vis spectrum λ_{max} nm (ϵ)	GC-MS after trimethylsilylation retention time in min; *m*/*z* (% relative intensity)
curcumin[a]	25.4	428 (58530) in MeOH	na
dihydrocurcumin[b]	24.6, 26.0[c]	376 (32000) and 285 (7500) in EtOH[b]	na
tetrahydrocurcumin[a]	23.3	281 (15100) in MeOH	23.4; 588 (M^+, 0.4), 573 (10), 365 (15), 323 (5), 223 (5), 209 (100), 193 (8), 179 (13), 73 (19)
hexahydrocurcumin[a]	11.4	282 (7400) in MeOH	21.5; 590 (M^+, 2), 500 (18), 291 (19), 278 (9), 251 (6), 249 (8), 235 (17), 209 (100), 195 (9), 193 (7), 179 (18), 73 (24)
octahydrocurcumin[a]	8.8, 9.1[c]	282 (~7400) in HPLC eluent	20.6, 21.3; 664 (M^+, 0.1), 574 (15), 484 (23), 324 (19), 275 (18), 262 (98), 247 (23), 235 (29), 209 (100), 179 (27), 73 (53)
demethoxycurcumin[d]	24.7	424 (54800) in MeOH	na
hexahydro-demethoxy-curcumin[e]	11.1	282 (~7100) in HPLC eluent	20.5; 560 (M^+, 5), 470 (43), 291 (15), 278 (8), 263 (11), 249 35, 235 (44), 209 (84), 195 (10), 193 (18), 179 (100), 73 (57)
octahydro-demethoxy-curcumin[a]	8.6, 8.9[c]	282 (~7100) in HPLC eluent	20.0, 20.3; 634 (M^+, 0.1), 544 (10), 454 (23), 386 (5), 324 (14), 275 (11), 262 (100), 258 (8), 247 (17), 235 (15), 232 (27), 209 (44), 192 (10), 179 (60), 73 (44)
bis-demethoxycurcumin[a]	24.0	416 (46400) in MeOH	na
hexahydro-bis-demethoxy-curcumin[a]	10.8	280 (6000) in MeOH	19.2; 530 (M^+, 0.01), 440 (19), 295 (8), 261 (18), 248 (43), 219 (13), 205 (11), 192 (15), 179 (100), 73 (51)
octahydro-bis-demethoxy-curcumin[a]	8.3, 8.7[c]	280 (~6000) in HPLC eluent	18.4, 19.0; 604 (M^+, 0.03), 514 (16), 424 (25), 356 (11), 294 (11), 258 (11), 232 (100), 205 (28), 192 (15), 179 (83), 73 (53)

[a] Obtained through chemical synthesis. [b] According to Uehara et al. (*29*). [c] Two isomers. [d] Isolated from turmeric. [e] Obtained through enzymatic reaction; MeOH, methanol; EtOH, ethanol; na, not applicable because curcumin, dihydrocurcumin, demethoxycurcumin, and bis-demethoxycurcumin could not be gas chromatographed even after trimethylsilylation.

deionized water to remove traces of unreacted aldehyde. Melting points were 179−181 °C for curcumin [lit. (*2*) 182−183 °C] and 224−225 °C for bis-demethoxycurcumin [lit. (*2*) 223−224 °C]. UV−vis spectra and HPLC retention times were identical with those of the authentic compounds isolated from turmeric (**Table 1**).

Hexahydrocurcumin was prepared by hydrogenation of tetrahydrocurcumin according to the method of Uehara et al. (*29*). A solution of 20 mg of tetrahydrocurcumin (54 mmol) in 20 mL of methanol was stirred with 10 mg of a Pd catalyst (5% Pd on charcoal) under a H_2 atmosphere at 20 °C for 5 h and filtered, and the filtrate was evaporated in vacuo. The colorless oily product contained one major peak (>95%) according to HPLC analysis with UV detection at 280 nm. The chemical structure of the synthetic hexahydrocurcumin was confirmed by GC-MS analysis of the trimethylsilylated product (**Table 1**). The same hydrogenation procedure was used to synthesize hexahydro-bis-demethoxycurcumin from bis-demethoxycurcumin, whereas hexahydrodemethoxycurcumin was obtained from demethoxycurcumin by enzymatic reduction with cytosol (see below) and purified by HPLC. The structures of both hexahydro compounds were also confirmed by GC-MS analysis (**Table 1**).

Octahydrocurcumin was obtained from 1 mL of a 2.5 mM solution of hexahydrocurcumin in methanol by adding a 10-fold molar excess of sodium borohydride. After 2 h at ambient temperature, the methanol was evaporated in vacuo and the residue dissolved in 1 mL of deionized water adjusted to pH 4.5. The product was extracted with 3 × 0.5 mL of ethyl acetate, the combined extract evaporated in vacuo, and the residue dissolved in 50 μL of methanol. Analysis by HPLC at 280 nm and by GC-MS after trimethylsilylation showed the presence of two products, which gave identical mass spectra consistent with the structure of octahydrocurcumin (**Table 1**). The two octahydrocurcumins represent diastereomers due to the presence of the two chiral carbon atoms carrying the aliphatic hydroxyl groups (**Figure 2**).

HPLC Analysis. A HP 1100 system equipped with a binary pump, a photodiode array detector, and HP Chemstation software for data collection and analysis (Agilent Technologies, Waldbronn, Germany) was used, and separation was carried out on a 250 × 4.6 mm i.d., 5 μm, reversed-phase Prodigy 5ODS^2 column (Phenomenex, Torrance, CA). Samples were dissolved in methanol, and the injection volume was 10−20 μL. A linear gradient changing from 30 to 70% acetonitrile (solvent B) in deionized water (solvent A, adjusted to pH 3.0 with formic acid) in 35 min was used. The flow rate was 1 mL/min. The curcuminoids were recorded at 420 nm and the reductive metabolites at 280 nm. 3,5,3′,5′-Tetramethyl-bisphenol A (TMBPA) was used as internal standard and monitored at 280 nm. Peaks were identified by comparison of HPLC retention times and absorption spectra with those of authentic standards (**Table 1**). Quantification was based on the peak area ratio (test compound to internal standard), applying different absorption coefficients for curcuminoids and their reductive metabolites (**Table 1**). The absorbance of hexahydrocurcumin and hexahydro-bis-demethoxycurcumin did not change upon reduction to octahydrocurcumin and octahydro-bis-demethoxycurcumin, respectively. Therefore, octahydrocurcuminoids were quantified by using the same absorption coefficient as for hexahydrocurcuminoids and by adding the peak areas of both diastereomers. The limits of quantification were 3 pmol for curcuminoids and 15 pmol for hexahydro- and octahydrocurcuminoids.

GC-MS Analysis. A Finnigan GCQ capillary gas chromatograph equipped with a 30 m × 0.25 mm i.d., 0.25 μm, 5% phenylmethyl MDN-5S fused-silica column (Supelco, Bellefonte, PA) and coupled to an ion-trap detector was operated with electron impact (EI) ionization at 70 eV (Thermo Finnigan, Austin, TX). Oven temperature was programmed from 150 °C (1-min hold) to 295 °C (10-min hold) at a rate of 10 °C/min. Temperatures of injector, transfer line, and ion source were 275, 275, and 250 °C, respectively. Samples (1 μL) dissolved in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were splitlessly injected with helium as carrier gas with a column pressure of 83 kPa. Mass spectra were scanned from *m*/*z* 50 to 650 at a rate of 0.5 s/scan. Metabolites of curcuminoids from the incubations were identified by comparison of their HPLC and GC retention times and their mass spectra with those of authentic reference substances.

Incubation of Liver Slices. Precision-cut slices were prepared from the livers of two male and two female untreated Sprague−Dawley rats as previously described (*30*). Two independent experiments were carried out for each of the three curcuminoids and both genders, using slices from different rats. For the reductive metabolites tetrahydrocurcumin and hexahydrocurcumin, two independent experiments with slices from one male and one female rat were conducted. For each experiment, three liver slices were separately incubated according to the method of Fisher et al. (*31*). The final concentration of the test compounds was

50 or 200 μM. Two types of control cultures, that is, one without liver slice and the other without test compound but with DMSO, were performed. Slices were incubated for 2, 4, 6, and 24 h. After incubation, the media of the three slices used in each experiment were combined and stored at −80 °C until analyzed.

Enzymatic Hydrolysis. Prior to workup, 10 μL of a 5 mM solution of TMBPA in DMSO was added as internal standard to each milliliter of the combined media. Two different methods of enzymatic hydrolysis were used.

Method I. For the determination of unconjugated metabolites (value IA), a 0.5-mL aliquot of the medium was extracted three times with 0.5 mL each of ethyl acetate. The combined extracts were evaporated in vacuo, the residue was dissolved in 50 μL of methanol,and a 10−20-μL aliquot was analyzed by HPLC. For measuring the sum of unconjugated and conjugated material (value IB), another 0.5-mL aliquot of the medium was mixed with 0.2 mL of 0.15 M acetate buffer (pH 5.0), incubated with 10 μL of β-glucuronidase/arylsulfatase preparation from *H. pomatia* at 37 °C for 16 h, extracted with ethyl acetate, and analyzed by HPLC as described above. The amount of conjugates was calculated as the difference between values IB and IA.

Method II of the enzymatic hydrolysis provided information about the type of conjugate by separate treatment of the incubation medium with β-glucuronidase and sulfatase. Unconjugated material (value IIA) was first determined in a 0.2-mL aliquot mixed with 0.2 mL of 0.15 M acetate buffer (pH 5.0) but no enzyme prior to extraction with ethyl acetate. Another 0.2-mL aliquot of the medium was mixed with 0.2 mL of 0.15 M acetate buffer (pH 5.0) and incubated with 5000 Fishman units (U) of β-glucuronidase type B-1 from bovine liver prior to extraction with ethyl acetate (value IIB). For the analysis of sulfates, 0.2 mL of medium was mixed with 0.2 mL of 0.1 M phosphate buffer (pH 7.1) containing 0.1 U of sulfatase type VI from *Aerobacter aerogenes* prior to incubation and extraction (value IIC). For total metabolites, that is, unconjugated, glucuronides, and sulfates, a 0.2-mL aliquot of the medium was mixed with 5000 Fishman U of β-glucuronidase type B-1 and 0.1 U of sulfatase type VI in 0.2 mL of 0.15 M acetate buffer (pH 5.0) (value IID). All enzymatic hydrolyses were carried out at 37 °C for 2 h, followed by extraction with ethyl acetate and analysis by HPLC as described above for method I. The difference between value IIB and value IIA gave the amount of glucuronides and the difference between value IIC and value IIA the amount of sulfates. The sum of unconjugated material, glucuronides, and sulfates derived from these calculations corresponded well with value IID, which was also used for comparison with value IB.

For confirmation by GC-MS analysis, the reductive metabolites tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin generated from curcumin in liver slices were extracted with ethyl acetate from the HPLC fractions obtained after enzymatic hydrolysis using method I. The extract was then evaporated in vacuo and the residue dissolved in methanol. Solvent was removed from an aliquot of the sample under a stream of nitrogen, and trimethylsilyl (TMS) ether derivatives were prepared by treatment with 25 μL of BSTFA at room temperature for at least 16 h.

Incubation of Liver Subcellular Fractions. Microsomes and cytosol were prepared from the livers of untreated male Sprague−Dawley rats as described previously by Lake (*32*). Protein concentrations were measured according to the method of Bradford (*33*) with bovine serum albumin as standard. The concentration of active cytochrome P450 (CYP) was determined by using the method of Omura and Sato (*34*).

Oxidative in vitro metabolism of curcuminoids was studied by incubating rat hepatic microsomes (1 mg of microsomal protein/mL) with 100 μM substrate dissolved in DMSO (final concentration = 1%) and a NADPH-generating system (0.9 U of isocitrate dehydrogenase, 9.4 mM isocitrate, 1.21 mM $NADP^+$, and 4.3 mM magnesium chloride) in a final volume of 1 mL in 0.1 M phosphate buffer (pH 7.4). After preincubation for 5 min at 37 °C, the NADPH-generating system was added and the mixture incubated for 40 min at 37 °C. Subsequently, the incubation mixture was extracted with 3 × 0.5 mL pf ethyl acetate and the extract evaporated to dryness. The residue was dissolved in 50 μL of methanol for HPLC analysis. Control incubations contained substrate but no NADPH-generating system. To ensure the activity of the microsomes, control incubations were also conducted with 100 μM testosterone and analyzed as described by Pfeiffer et al. (*30*).

For reductive in vitro metabolism, incubations were conducted in a final volume of 0.5 mL containing a 100 μM mixture of curcuminoids from turmeric (75% curcumin, 18% demethoxycurcumin, and 7% bisdemethoxycurcumin) dissolved in DMSO (final concentration = 1%), equine alcohol dehydrogenase (5 U) or rat hepatic cytosol (1 mg of cytosolic protein/mL), and 10 mM NADH in 0.1 M phosphate buffer (pH 7.4). After a preincubation period of 5 min at 37 °C, NADH was added and the mixture incubated for 1 h at 37 °C. The reaction was terminated by extraction with 3 × 0.5 mL of ethyl acetate, the extract evaporated to dryness in vacuo, the residue dissolved in 50 μL of methanol, and an aliquot used for HPLC analysis. In control experiments, the curcuminoid mixture was incubated with (i) the cytosol but without NADH and (ii) NADH without equine alcohol dehydrogenase.

In vitro reduction and glucuronidation of curcumin were studied separately and in combination, using liver cell fractions from male Sprague−Dawley rats. Incubations were performed in 0.1 M phosphate buffer (pH 7.4) containing 100 μM curcumin dissolved in DMSO (final concentration = 1%) and 10 mM magnesium chloride in a total volume of 1 mL. For reduction, incubation mixtures included 1 mg of cytosolic protein/mL and 10 mM NADH. For glucuronidation, 1 mg of microsomal protein/mL and 4 mM UDPGA were added. For simultaneous reduction and glucuronidation, cytosol and microsomes were combined and their cofactors added. After 5 min of preincubation of cell fractions and substrates at 37 °C, reactions were started by the addition of the cofactor(s) and incubations continued for 1 h at 37 °C. Control incubations were carried out with cytosol and microsomes but without NADH or UDPGA or both cofactors. Reactions were terminated by adding 25 μL of 20% aqueous trichloroacetic acid. Subsequently, the incubation mixture was neutralized with 25 μL of 1 M aqueous sodium hydroxide, and the precipitated proteins were removed by centrifugation (5 min at 1000*g*). The resulting supernatant was analyzed by HPLC directly and after enzymatic hydrolysis in a 0.2-mL aliquot with a β-glucuronidase/arylsulfatase preparation from *H. pomatia* in 0.2 mL of 0.15 M acetate buffer (pH 5.0) as described for method I above.

RESULTS AND DISCUSSION

Metabolism of Curcuminoids in Liver Slices. Freshly prepared liver slices from male and female Sprague−Dawley rats were incubated with 200 μM curcumin for 24 h and the culture media analyzed for curcumin and its phase I and phase II metabolites. Authentic reference compounds were available for the reductive curcumin metabolites tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin, which differed in their HPLC retention times (**Table 1**).

A representative HPLC profile of the phase I metabolites of curcumin extracted from the incubation medium of a male liver slice after hydrolysis of the conjugates is depicted in **Figure 3**. The absorbance at 280 nm was selected because the known reductive metabolites of curcumin do not absorb at 420 nm (**Table 1**). In addition to curcumin, two major peaks were observed, which eluted after 11.4 and 9.1 min. The peak at 11.4 min coeluted with synthetic hexahydrocurcumin and had an identical UV−vis spectrum. When the HPLC peak was collected and the metabolite extracted and analyzed by GC-MS, the GC retention time and mass spectrum were the same as for authentic hexahydrocurcumin. Likewise, the peak at 9.1 min coeluted with one of the diastereomers of synthetic octahydrocurcumin, whereas the other authentic diastereomer had a HPLC retention time of 8.8 min and cochromatographed with the small peak preceding the peak at 9.1 min in **Figure 3**. Collection of the HPLC eluate from 8.5 to 9.5 min and analysis by GC-MS confirmed the presence of the two diastereomers of octahydrocurcumin in a ratio of 1:3. The HPLC peaks at 11.4 and 9.1 min were not detectable in control incubations containing no

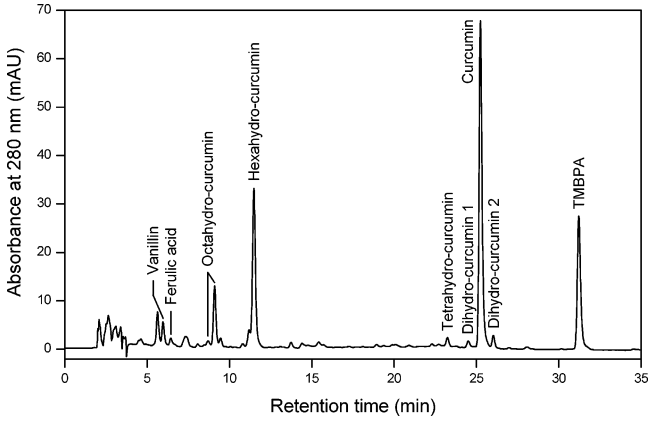

Figure 3. HPLC profile of the phase I metabolites of curcumin extracted from the medium of a 24-h incubation of 200 μM curcumin with the slice of a male Sprague–Dawley rat liver after conjugate hydrolysis with β-glucuronidase/arylsulfatase from *H. pomatia*. TMBPA, internal standard.

curcumin or no liver slice. However, the incubation of curcumin in the absence of a liver slice gave rise to the formation of vanillin and ferulic acid, which are known products of the chemical decomposition of curcumin (*35*) and which were also observed in the HPLC profile of the complete incubation (**Figure 3**).

Of the other minor peaks visible in **Figure 3**, only those eluting after 23.3, 24.6, and 26.0 min were not present in the HPLC profile of control incubations. The peak at 23.3 min cochromatographed with synthetic tetrahydrocurcumin and had the same UV−vis spectrum and mass spectrum upon GC-MS analysis. The peaks at 24.6 and 26.0 min were tentatively identified as isomers of dihydrocurcumin on the basis of their UV−vis spectra, which were identical and corresponded to the published spectrum for dihydrocurcumin in ethanol (*29*). The formation of two isomers is probably due to the reduction of either the allylic or the enolic double bond of curcumin (see **Figure 1**).

No hints for oxidative metabolites of curcumin, for example, products of aromatic hydroxylation or demethylation, were obtained from the HPLC profiles, even when the detector was set to 420 nm, which should be near the maximum of absorption of oxidative metabolites due to the presence of the curcumin chromophore. On the basis of the limit of detection (LOD) at this wavelength, it is estimated that oxidative curcumin metabolites would have been observed if at least 0.01% of the curcumin were converted to such products.

When demethoxycurcumin and bis-demethoxycurcumin were incubated with rat liver slices under the same conditions as curcumin and the incubation media analyzed by HPLC, the pattern of their phase I metabolites was very similar to that of curcumin: the major metabolites were the hexahydro and octahydro products, which were unambiguously identified by cochromatography with the respective reference compounds in HPLC and GC-MS analysis (**Table 1**). The other phase I metabolites of demethoxycurcumin and bis-demethoxycurcumin were tentatively identified as two dihydro and one tetrahydro product, on the basis of their HPLC retention times and UV−vis spectra. No evidence for the formation of oxidative metabolites of the demethoxylated curcuminoids was provided by HPLC analysis.

A quantitative account of the phase I metabolites and their extent of conjugation in liver slices from male and female rats is given in **Figure 4** for all three curcuminoids. As enzymatic hydrolysis with β-glucuronidase/arylsulfatase (method I) was

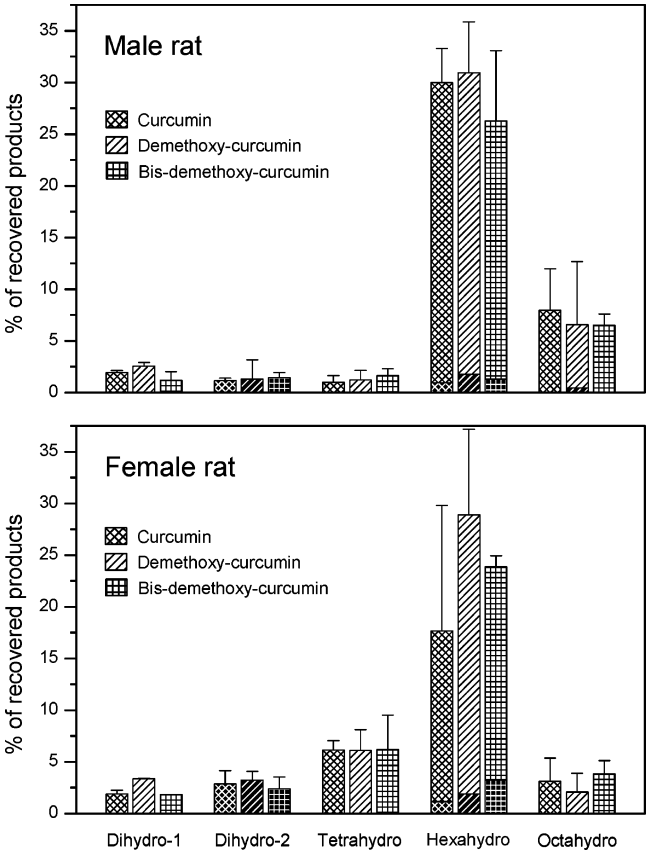

Figure 4. Pattern of reductive metabolites of curcumin, demethoxycurcumin, and bis-demethoxycurcumin in incubation media of liver slices from male (top) and female (bottom) Sprague–Dawley rats after 24 h of incubation with curcuminoids. For each metabolite, the proportion of unconjugated (darker part of bar) and conjugated (lighter part) material was determined using hydrolysis with β-glucuronidase/arylsulfatase from *H. pomatia* (method I). Data are expressed as percent of the sum of recovered curcuminoid and metabolites and represent the mean of two rats for each gender and three independently incubated slices per rat liver.

used, the proportion of conjugates indicated in each column of **Figure 4** represents the sum of glucuronides and sulfates. The conjugated hexahydro product was the predominating metabolite of all three curcuminoids in liver slices from both genders, whereas the two isomers of the dihydro metabolites were formed only to a small extent. Interestingly, one isomer was completely conjugated, whereas the other isomer appeared to be a poor substrate for conjugation. The phase I metabolism of the three curcuminoids suggested a slight gender difference: male rats generated more octahydro metabolites but fewer tetrahydro metabolites than female rats; all of these metabolites were present as conjugates (**Figure 4**).

In addition to the metabolites, the amounts of parent curcuminoids were quantified in the 24-h incubations with liver slices (**Table 2**). Most notably, the parent compounds were present only in unconjugated form, and the extent of metabolic conversion of parent curcuminoids to reductive and conjugated metabolites was in the range of 30−50%. When the sums of recovered parent curcuminoids and their reductive metabolites were compared to the amounts of curcuminoids added to the incubations, it became apparent that major portions of the incubated curcuminoids could not be accounted for. The recoveries were only ∼50% for curcumin and demethoxycurcumin but ∼75% for bis-demethoxycurcumin with both male

Table 2. Recoveries of Parent Curcuminoids and Their Reductive Metabolites (Unconjugated and Conjugated Forms) from Incubations with Male and Female Rat Liver Slices after Conjugate Hydrolysis with β-Glucuronidase/Arylsulfatase from *H. pomatia*

compound	rat gender	parent compound	reductive metabolites	total recovery	metabolic conversion[a]
curcumin	male	27.9 ± 3.2	25.8 ± 2.9	53.7 ± 6.1	48.0 ± 0.1
	female	29.7 ± 11.6	12.1 ± 1.1	41.8 ± 10.5	31.6 ± 10.7
demethoxycurcumin	male	30.5 ± 7.9	22.2 ± 1.3	52.7 ± 9.2	43.1 ± 5.1
	female	26.1 ± 6.6	20.1 ± 0.5	46.2 ± 7.1	44.3 ± 5.7
bis-demethoxycurcumin	male	48.8 ± 16.0	27.4 ± 1.1	76.3 ± 17.1	37.5 ± 7.0
	female	46.4 ± 9.7	28.2 ± 4.1	74.6 ± 13.8	38.1 ± 1.5

[a] Metabolic conversion: percent of total recovered material present as reductive and conjugated metabolites. Data are expressed as percent of the administered amount of curcuminoid and represent the mean ± range of two independent experiments.

Table 3. Pattern of Conjugates in the Medium of Liver Slices from Male and Female Rats after 24 h of Incubation with Curcumin, Tetrahydocurcumin, and Hexahydrocurcumin

		curcumin (200 μM)		tetrahydrocurcumin (200 μM)		hexahydrocurcumin (50 μM)	
compound		male	female	male	female	male	female
curcumin	unconjugated	26.3 ± 5.7	50.6 ± 14.0	na	na	na	na
	glucuronide	16.5 ± 1.2	13.2 ± 1.3	na	na	na	na
	sulfate	9.2 ± 2.5	6.7 ± 0.6	na	na	na	na
tetrahydrocurcumin	unconjugated	nd	nd	4.4 ± 0.4	5.9 ± 1.0	na	na
	glucuronide	0.9 ± 0.1	5.5 ± 0.1	2.0 ± 0.1	9.5 ± 0.8	na	na
	sulfate	nd	nd	nd	nd	na	na
hexahydrocurcumin	unconjugated	0.9 ± 0.9	2.0 ± 1.0	8.3 ± 0.4	3.8 ± 0.2	nd	nd
	glucuronide	31.9 ± 6.7	15.2 ± 7.9	70.8 ± 1.8	74.9 ± 0.5	42.2 ± 2.7	76.8 ± 1.2
	sulfate	4.1 ± 0.4	2.2 ± 1.6	3.5 ± 0.6	4.3 ± 1.8	14.6 ± 1.7	19.6 ± 2.5
octahydrocurcumin	unconjugated	nd	nd	2.0 ± 0.4	nd	8.6 ± 2.0	2.1 ± 2.1
	glucuronide	6.5 ± 2.1	0.7 ± 0.7	9.0 ± 2.7	1.7 ± 1.7	34.6 ± 1.0	1.6 ± 1.6
	sulfate	nd	nd	nd	nd	nd	nd

[a] na, not applicable; nd, not detectable, <0.03% for tetrahydrocurcumin and <0.25% for hexahydrocurcumin and octahydrocurcumin. The limit of quantification was the 3-fold value of the LOD. Data are expressed as percent of all detected compounds (parent compound plus metabolites) and represent the mean ± range of a duplicate determination of three independently incubated slices per rat.

and female liver slices. Thus, about half of the curcumin and demethoxycurcumin, but only 25% of the bis-demethoxycurcumin, administered to the liver slices had "disappeared". About the same losses were noted when the individual curcuminoids were incubated for 24 h with the medium in the absence of liver slices. Disappearance of curcumin and demethoxycurcumin and, to a lesser extent, bis-demethoxycurcumin was also observed when the curcuminoid mixture from turmeric, consisting of 75% curcumin, 18% demethoxycurcumin, and 7% bis-demethoxycurcumin, was incubated for 24 h without liver slices, resulting in a shift of the composition from curcumin toward bis-demethoxycurcumin. The observed loss of curcuminoids is in agreement with previous studies showing that these compounds are unstable in aqueous solution, with curcumin being the least and bis-demethoxycurcumin the most stable curcuminoid (*35*, *36*). Indeed, when the 24-h incubations of curcumin were analyzed by HPLC for the presence of known degradation products, vanillin and ferulic acid were detected both in the absence and presence of liver slices (**Figure 3**).

The issue of chemical instability of curcuminoids raises questions about the incubation conditions, in particular, with respect to the incubation time. Pilot studies with slices from male rat liver showed that short-term incubations of 2, 4, and 6 h gave rise to the same pattern of phase I and II metabolites as observed after 24 h, but the sum of metabolites was smaller and the proportion of unmetabolized curcumin higher, as was the total recovery. For example, the metabolic conversion rate was 17% and the recovery 69% in 6-h incubations as compared with 48 and 54%, respectively, in 24-h incubations. Concentrations of curcumin of <200 μM resulted in lower recoveries of metabolites and parent compound.

Detailed Investigation of Curcumin Metabolism in Liver Slices. To further study the reductive metabolites and conjugates of curcumin in rat liver slices, 24-h incubations of curcumin, tetrahydrocurcumin, and hexahydrocurcumin were conducted in liver slices of male and female Sprague−Dawley rats. A modification of the enzymatic hydrolysis was used, involving separate hydrolysis with β-glucuronidase and sulfatase and a hydrolysis time of only 2 h (method II). The results of these experiments are summarized in **Table 3**.

With 200 μM curcumin, the major phase I metabolites again were hexahydrocurcumin, tetrahydrocurcumin, and octahydrocurcumin. As in the earlier study (**Figure 4**), liver slices from male rats generated more octahydrocurcumin and liver slices from female rats more tetrahydrocurcumin. The preferred pathway for conjugation of all three major reductive curcumin metabolites was glucuronidation, but sulfates were also formed in significant amounts (**Table 3**). Large amounts of the glucuronide and sulfate were also found for curcumin itself, which is in clear contrast to the earlier study in which the parent curcuminoids were present in the 24-h incubations only in unconjugated form.

When 200 μM tetrahydrocurcumin was incubated for 24 h, little parent compound but large amounts (>80%) of hexahydrocurcumin together with small quantities of octahydrocurcumin were found in the incubation media of male and female slices (**Table 3**). Again, male rat liver formed more octahydro metabolites than female liver, and glucuronides were predomi-

Table 4. Metabolic Studies with Microsomes and Cytosol from the Liver of Male Sprague–Dawley Rats and with Alcohol Dehydrogenase from Equine Liver

metabolic system	substrate	workup	result of HPLC analysis[d]
microsomes/NADPH[a]	curcumin from turmeric[b]	extraction[c]	parent curcuminoids, no oxidative metabolites, traces of hexahydrocurcuminoids
alcohol dehydrogenase/NADH	curcumin from turmeric[b]	extraction[c]	reductive metabolites of curcuminoids (hexahydro > tetrahydro ≅ octahydro ≫ dihydro-1 and dihydro-2)
cytosol/NADH	curcumin from turmeric[b]	extraction[c]	reductive metabolites of curcuminoids (hexahydro > tetrahydro ≅ octahydro ≫ dihydro-1 and dihydro-2)
microsomes/UDPGA	pure curcumin	precipitation of proteins	large peaks of curcumin and of curcumin-glucuronide (at 16.6 min)
microsomes/UDPGA	pure curcumin	precipitation of proteins, then hydrolysis by method I	large peak of curcumin, but less than the sum of curcumin and curcumin-glucuronide detected prior to hydrolysis
cytosol/NADH plus microsomes/UDPGA	pure curcumin	precipitation of proteins	large peaks of curcumin and curcuminglucuronide, small peak of unconjugated hexahydrocurcumin
cytosol/NADPH plus microsomes/UDPGA	pure curcumin	precipitation of proteins, then hydrolysis by method I	large peaks of curcumin and of hexahydro-, tetrahydro-, and octahydrocurcumin

[a] NADPH-generating system. [b] Consisting of 75% curcumin, 18% demethoxycurcumin, and 7% bis-demethoxycurcumin. [c] With ethyl acetate. [d] LOD was 1 pmol for curcuminoids, 3 pmol for tetrahydrocurcuminoids, and 5 pmol for hexahydro- and octahydrocurcuminoids.

nating. The metabolic conversion rate and total recovery of parent compound plus metabolites were much higher for tetrahydrocurcumin (>90%) than for curcumin (~50%).

The higher capacity of the male versus the female rat liver for the reduction of hexahydrocurcumin to octahydrocurcumin was again obvious when 50 μM hexahydrocurcumin was incubated for 24 h (**Table 3**): 90% of hexahydrocurcumin, although mostly glucuronidated, was still present in slices from female rat liver, but only ~50% was found for males. Total recoveries were near 100% for both genders. The comparison of the metabolism of tetrahydrocurcumin and hexahydrocurcumin emphasizes the fact that tetrahydrocurcumin is avidly reduced to hexahydrocurcumin by both male and female rat liver, although female liver is somewhat less efficient. The further reduction to octahydrocurcumin is less efficient in both genders, especially in females. Glucuronidation is preferred over sulfation for the conjugation of the reductive metabolites. Furthermore, hints for a sulfoglucuronide of hexahydrocurcumin were obtained: when the amounts of hexahydrocurcumin released by separate hydrolysis with β-glucuronidase and sulfatase were added, the sum was always less than the amount of hexahydrocurcumin liberated by hydrolysis with a mixture of the two enzymes (data not shown).

Metabolism of Curcuminoids in Subcellular Fractions. Because no products of aromatic hydroxylation or demethylation were observed in liver slices, the ability of rat liver to form oxidative metabolites of curcuminoids was probed with rat hepatic microsomes. Furthermore, because of the observed discrepancy in the amount of curcumin conjugates found after enzymatic cleavage with β-glucuronidase/arylsulfatase from *H. pomatia* (hydrolysis method I: no conjugated curcumin observed) and with pure β-glucuronidase and sulfatase (hydrolysis method II: glucuronides and sulfates of curcumin observed), the glucuronidation of curcumin and its reductive metabolites was studied with rat hepatic microsomes. The experiments and corresponding results are listed in **Table 4**.

When microsomes were incubated with a mixture of the three curcuminoids in the presence of a NADPH-generating system for 40 min, only unchanged curcuminoids and traces of the hexahydro metabolites were detectable in the ethyl acetate extract by HPLC with diode array detection, using detector wavelengths suitable for both curcumin and its reductive metabolites. Our HPLC method would have detected if 0.01% of curcumin were converted to an oxidative metabolite, assuming the same absorbance as for curcumin. For reductive metabolites, the LOD would have indicated a conversion of 0.05% of the administered curcumin. Thus, neither oxidative nor reductive curcumin metabolites were formed to any significant extent by rat liver microsomes. The monooxygenese activity of the microsomes was ensured by incubations with testosterone, which gave rise to the expected pattern of oxidative testosterone metabolites as described previously by Pfeiffer et al. (*30*).

In contrast to the failure to observe oxidative or reductive curcumin metabolites in microsomal incubations, the full spectrum of reductive phase I metabolites was formed when curcuminoids were incubated with an alcohol dehydrogenase preparation or with rat liver cytosol in the presence of NADH (**Table 4**).

For the experiments on microsomal glucuronidation, pure curcumin was used, and the incubation medium after precipitation of proteins was analyzed by HPLC without extraction to avoid the loss of glucuronides. Incubation of microsomes with curcumin in the presence of UDPGA clearly gave rise to a novel product eluting earlier than curcumin from the HPLC column and most likely representing a glucuronide of curcumin, because it disappeared after hydrolysis with β-glucuronidase/arylsulfatase from *H. pomatia* (**Table 4**). However, the area of the curcumin peak observed after hydrolysis was clearly smaller than the sum of the areas for the curcumin peak and curcumin-glucuronide peak determined prior to hydrolysis. When curcumin was incubated with cytosol plus microsomes and the respective cofactors, curcumin-glucuronide together with small amounts of unconjugated reductive curcumin metabolites were detected by HPLC prior to hydrolysis; after cleavage of conjugates, the peaks of the reductive metabolites increased markedly. It is assumed that the reductive metabolites are extensively glucuronidated but that these glucuronides eluted with the solvent front under the HPLC conditions used.

Conclusion. Our study on the metabolism of curcumin in precision-cut liver slices from male and female Sprague–Dawley rats and with hepatic microsomes and cytosol has confirmed earlier reports from other laboratories (*16*, *20*, *21*, *37*) that reduction of the aliphatic moiety is the only pathway in phase I metabolism. No evidence for the formation of hydroxylation or demethylation products of curcumin was

obtained in our experiments. Although no reference substances for such oxidative curcumin metabolites were at hand, they should have been detectable in our HPLC system due to their putative similarity of absorbance with curcumin and their expected retention times, which should be somewhat shorter than that of curcumin on the reversed-phase column. The major reductive curcumin metabolite formed in rat liver slices and in hepatic cytosol was hexahydrocurcumin, together with smaller amounts of tetrahydrocurcumin and octahydrocurcumin. This is consistent with the report by Holder et al. (*16*) that glucuronides of hexahydrocurcumin and tetrahydrocurcumin are the major biliary metabolites after intraveneous injection of tritium-labeled curcumin to bile duct-cannulated male Sprague−Dawley rats. After intraperitoneal injection of curcumin into female BALB/c mice, glucuronides of tetrahydrocurcumin and dihydrocurcumin were the major phase I metabolites in plasma (*37*). Ireson et al. (*20*) reported that curcumin is metabolized to hexahydrocurcumin and octahydrocurcumin in hepatocytes from male and female Fischer 344 rats. This laboratory also studied the reductive metabolism of curcumin in more detail and observed that cytosol or alcohol dehydrogenase is required for the formation of tetrahydrocurcumin and hexahydrocurcumin, but microsomes are needed for the reduction of hexahydrocurcumin to octahydrocurcumin (*21*).

No reports have been published in the literature to date on the phase I metabolism of demethoxycurcumin and bis-demethoxycurcumin. Our study has demonstrated for the first time that these curcuminoids follow the same pathways as curcumin: no oxidative metabolites were observed, and the pattern of the reductive products was about the same as obtained with curcumin. For the major reductive metabolites of demethoxycurcumin and bis-demethoxycurcumin, the EI mass spectra obtained by GC-MS analysis of the trimethylsilylated metabolites provided characteristic features suitable for unambiguous identification (**Table 1**). For example, the mass spectra of the hexahydro metabolites of all three curcuminoids exhibit very small molecular ions and the loss of 90 mass units from the molecular ions, which is due to the elimination of trimethylsilanol. Other characteristic and abundant ions in the mass spectra arise from cleavage of the benzylic bonds. The mass spectra of the octahydrocurcuminoids indicate two successive eliminations of trimethylsilanol, together with fragmentations at the benzylic positions. Another prominent fragmentation of the octahydrocurcuminoids, leading to *m*/*z* 262 and 232, occurs in the aliphatic chain after the loss of the two trimethylsilanol molecules.

In addition to bioreduction, curcumin and its reductive metabolites undergo conjugation with glucuronic acid and sulfate in liver slices. The formation of such conjugates has been reported by others before, for example, by Holder et al. (*16*) in the bile of bile duct-cannulated rats, by Pan et al. (*37*) in the blood plasma of mice, and by Ireson et al. (*20*) in rats in vivo and rat hepatocytes in vitro. The evidence for the type of conjugate was mostly based on cleavage with either β-glucuronidase or sulfatase, but in some cases mass spectra of the glucuronide or sulfate were obtained by LC-MS/MS (*20*, *21*, *37*).

In our study, no conjugates of the parent curcuminoids but large amounts of conjugates of the reductive metabolites were observed when the hydrolysis of conjugates was conducted with a β-glucuronidase/arylsulfatase preparation from *H. pomatia* for 16 h (**Figure 4**). However, significant amounts of glucuronides and sulfates were found both of the reductive metabolites and of parent curcumin when glucuronides and sulfates were separately hydrolyzed by β-glucuronidase and sulfatase, respectively, for 2 h only (**Table 3**), in agreement with the formation of curcumin-glucuronide and curcumin-sulfate reported by others (*19*−*21*, *37*). This discrepancy is most likely due to the chemical instability of curcumin. The spontaneous time- and pH-dependent degradation of curcumin in aqueous systems has been previously described by Wang et al. (*35*). It has also been reported that curcumin was more stable in aqueous media in the presence of fetal calf serum and in blood than in pure buffer (*35*, *36*). Obviously, the curcumin released from its glucuronide and sulfate in our study had completely decomposed after 16 h of hydrolysis but was still present to a significant extent after 2 h of hydrolysis. In contrast to curcumin, a large proportion of the major reductive metabolites was found in the 16-h incubation, indicating their higher chemical stability as compared to curcumin. It has previously been reported by Pan et al. (*37*) that tetrahydrocurcumin is much more stable in aqueous buffer than curcumin.

The instability of free curcumin may be one of the reasons for the poor recovery observed in our incubations of the curcuminoids, which were about 50% for curcumin and demethoxycurcumin and 75% for bis-demethoxycurcumin. The better recovery for bis-demethoxycurcumin implies that this curcuminoid has a higher chemical stability, which has also been reported earlier (*36*).

The chemical instability of curcumin may also explain, at least in part, the failure of clinical studies to detect significant concentrations of curcumin in the plasma and urine of patients even after heroic doses of curcumin. For example, maximum plasma levels of only 1.8 μM curcumin were observed after an oral dose of 8 g of curcumin, and no curcumin was detectable in urine (*22*). Following oral doses of 180 mg of curcumin for up to 4 months, no curcumin was detectable in plasma or urine (*23*). In a more recent study by Sharma et al. (*25*) with colorectal cancer patients receiving daily oral doses of 3.6 g of curcumin for up to 4 months, maximum blood plasma levels in the range of 11, 16, and 9 nmol/L were measured for curcumin, curcumin-glucuronide, and curcumin-sulfate, respectively. In the urine of the same patients, maximum concentrations of curcumin, curcumin-glucuronide, and curcumin-sulfate were on the order of 1, 500, and 40 nmol/L. From the very low levels of curcumin and its conjugates in blood and urine, it is currently concluded that curcumin possesses poor systemic bioavailability after oral dosing. To corroborate this assumption, it is proposed to use the urinary excretion of the glucuronides of hexahydrocurcumin and octahydrocurcumin as markers for systemic bioavailability in future clinical studies. These major metabolites of curcumin have the distinct advantage of being much more chemically stable than curcumin and will also account for the extensive metabolic bioreduction of this important curcuminoid.

ABBREVIATIONS USED

BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; NADH, nicotinamide dinucleotide, reduced form; NADPH, nicotinamide dinucleotidephosphate, reduced form; TMS, trimethylsilyl; U, enzyme unit; UDPGA, uridine-5′-diphosphoglucuronic acid; LOD, limit of detection.

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