Comparison of systemic availability of curcumin with that of curcumin formulated with phosphatidylcholine

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Abstract *Purpose:* Curcumin, a major constituent of the spice turmeric, suppresses expression of the enzyme cyclooxygenase 2 (Cox-2) and has cancer chemopreventive properties in rodents. It possesses poor systemic availability. We explored whether formulation with phosphatidylcholine increases the oral bioavailability or affects the metabolite profile of curcumin. *Methods:* Male Wistar rats received 340 mg/Kg of either unformulated curcumin or curcumin formulated with phosphatidylcholine by oral gavage. Rats were killed at 15, 30, 60 and 120 min post intubation. Plasma, intestinal mucosa and liver were analysed for the presence of curcumin and metabolites using HPLC with UV detection. Identity of curcumin and metabolites was verified by negative ion electrospray liquid chromatography/tandem mass spectrometry. *Results:* Curcumin, the accompanying curcuminoids desmethoxycurcumin and bisdesmethoxycurcumin, and the metabolites tetrahydrocurcumin, hexahydrocurcumin, curcumin glucuronide and curcumin sulfate were identified in plasma, intestinal mucosa and liver of rats which had received formulated curcumin. Peak plasma levels and area under the plasma concentration time curve (AUC) values for parent curcumin after administration of formulated curcumin were five-fold higher than the equivalent values seen after unformulated curcumin. Similarly, liver levels of curcumin were higher after administration of formulated curcumin as compared to unformulated curcumin. In contrast, curcumin concentrations in the gastrointestinal mucosa after ingestion of formulated curcumin were somewhat lower than those observed after administration of unformulated curcumin. Similar observations were made for curcumin metabolites as for parent compound. *Conclusion:* The results suggest that curcumin formulated

with phosphatidylcholine furnishes higher systemic levels of parent agent than unformulated curcumin.

Introduction

The incidence of cancer continues to rise as a consequence of an increasingly aging population. Improved identification of individuals at risk of developing the disease has increased the feasibility of employing long-term chemoprevention strategies in cancer management. Constituents of the diet are considered to be a promising source of novel efficacious and safe cancer chemopreventive agents [18]. One dietary polyphenol which has been the focus of considerable preclinical and early clinical chemoprevention studies is curcumin, [1,7-bis(4- hydroxyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. Curcumin is the major constituent of the spice turmeric extracted from the root of *Curcuma longa* Linn. Curcumin is a powerful antioxidant and inhibits the expression of the enzyme cyclooxygenase 2 (Cox 2) at least in part *via* interference with activation of the transcription factor NFkB [2, 14]. *In vitro*, curcumin inhibits the growth of cancer cells with an IC_{50} value of 20- 75μM [6, 16]. In rodent models, curcumin has been shown to prevent cancer in the colon, skin, stomach, duodenum, soft palate, tongue, sebaceous glands and breast [9, 10, 15]. Curcumin undergoes avid metabolism by conjugation (glucuronidation and sulfation) and reduction pathways [7, 8]. Clinical pilot studies have associated curcumin consumption with regression of pre-malignant lesions of bladder, soft palate, stomach, cervix and skin [1, 11]. Preclinical and clinical pilot studies suggest that concentrations of curcumin achieved in plasma and target tissues are low, probably caused, at least in part, by its extensive metabolism [4, 5, 7, 8]. In a phase I trial, plasma and urine concentrations of curcumin in patients, who had ingested 3600 mg curcumin orally, were 11.1 nmol/L and 1.3 μmol/L, respectively [17].

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Curcumin concentrations in colorectal tissues of patients on this dose were 7.7-12.7 nmol/g, whilst levels in the liver were below the limits of detection [4, 5]. In another study, peak plasma concentrations 1-2 h after oral dosing, reached 0.41-1.75 μM in patients receiving 4 to 8g curcumin [1]. It is neither practical nor desirable to increase the oral dose of curcumin above that already investigated. Formulating poorly absorbed drugs with phosphatidylcholine has previously been shown to increase their plasma bioavailability. For example, the anti-schistosomal activity of praziquantel was increased by incorporation into phosphatidylcholine-containing liposomes [13] and the intestinal permeability of hexarelin was increased 20-fold by such a formulation strategy [3]. Mindful of these facts, we tested the hypothesis that a formulation of curcumin with soy phosphatidylcholine might improve the systemic availability of curcumin in plasma and tissues in rat.

Materials and methods

Chemicals

Curcumin (CAS 458-37-7) and curcumin formulated with EpiKuron 130 P (30% phosphatidylcholine) were supplied by Indena spa (Milan, Italy). The formulated curcumin contained 16.89% curcuminoids, of which 93.82% was curcumin, and the ratio of curcumin to Epikuron 130 P was 1:4. Commercially available curcumin obtained by extraction of *Curcuma spp.* contains 94% curcuminoids of which 77% was curcumin, 17% desmethoxycurcumin and 6% bisdesmethoxycurcumin as determined by hplc, (Fig. 2A , for structures see Fig. 1). Tetrahydrocurcumin and hexahydrocurcumin were synthesized as described previously [19] and were kindly provided by Dr I Rubin, Phytopharm plc (Cambridge, UK). Dosing suspensions were prepared in 1% methylcellulose at 17 mg curcumin/mL. Experiments were carried out under animal project license PPL 40/2496, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCCR for animal welfare [19]. Male Wistar albino rats (250 g) were purchased from Harlan UK Ltd (Bicester, UK) and kept under a twelve-hour light/dark cycle on standard lab chow. Animals were fasted overnight and received unformulated or formulated curcumin at 340 mg/Kg (in terms of curcumin) by oral gavage. At 15, 30, 60 and 120 min animals were exsanguinated under terminal anaesthesia. Group size was 3 rats per time point. Whole blood was collected by cardiac puncture into heparinized tubes, centrifuged immediately at 7000xg for 15 min, plasma was then decanted and stored at -80°C until analysis. Liver and gastrointestinal tract from stomach to anus were removed. The intestinal tract was flushed with phosphate buffered saline, dissected longitudinally and then washed a second time to remove residual content. Mucosa was collected from small intestine and colon by scraping gently with a spatula. Liver and mucosa were flash-frozen in liquid nitrogen and stored at -80°C.

Sample preparation

Curcumin and curcumin metabolites were extracted from plasma by solid phase extraction. Plasma (1 mL) was loaded onto a 1cc Oasis HLB cartridge (Waters, Elstree, UK), washed with 25:25:1 methanol:water:glacial acetic acid (1 mL), and eluted with 1 mL of methanol containing 2% glacial acetic acid. Eluant was evaporated to dryness at 45°C under a stream of nitrogen, and the residue was re-suspended in 75μL of 50% aqueous acetonitrile. Standard solutions of curcumin (5-1000 ng/mL) were prepared in 1 mL human plasma (obtained from the National Blood Transfusion Centre, Sheffield, UK) and extracted as described above. Extraction efficiency was 59% with 2.5 and 4.5% intra and inter day variability, 99% accuracy and response was linear over the range 5-1000 ng/mL with an R^2 value consistently of 0.999.

Mucosa (100 mg) was suspended in 1.15% KCl (1 mL) and centrifuged (16,000xg, 60 sec). The pellet was re-suspended in 1.15% KCl (1 mL) and homogenized using a blade homogeniser set at top speed for 2 x 20 sec. Aliquots (0.1 mL) of homogenate were mixed with an equal volume of acetone:formic acid (9:1), the mixture was vortexed and kept at -20°C for 30 min prior to centrifugation (16,000xg, 5 min). The supernatant was decanted and evaporated to dryness at 45°C under a stream of nitrogen. The residue was re-suspended in 75 μL of 50 % aqueous acetonitrile prior to analysis.

Liver was homogenized 1:4 in isotonic KCl. An aliquot (0.5 mL) of liver homogenate was mixed with 2 mL of acetone:formic acid (9:1), and the mixture was immediately vortexed. Samples were kept at -20°C for 30 min prior to centrifugation (16,000xg, 10 min). Supernatant was evaporated to dryness at 45°C under a stream of nitrogen, and the residue was re-suspended in 75µL of 50% aqueous acetonitrile prior to analysis.

HPLC analysis

Analysis of samples was performed using a Varian Prostar series hplc instrument comprising a model 230 pump and a model 410 autosampler. Separation was achieved with an Atlantis dC18 column (4.6 x 150mm, 3µm, Waters, Elstree, UK) with a guard (4.6 x 20 mm, 3µm), kept at 35°C. The mobile phase consisted of two components: A, 10 mM ammonium acetate pH 4.5; B, acetonitrile. Initial conditions were 95% A progressing to 55% A at 20 min and 5% A at 33 min. The flow rate was 1.5 mL/min. Curcumin and conjugated metabolites were detected at 426 nm and reduced curcumin metabolites at 280 nm using a Varian 325 UV-vis detector.

LC/MS/MS analysis

The identity of curcuminoids was verified by negative ion electrospray tandem mass spectrometry employing multiple reaction monitoring (MRM). Analysis was performed using an API 2000 LC/MS/MS (Applied Biosystems MDS Sciex, Warringon, UK) equipped with an Agilent 1100 series sample delivery system. Separation of curcumin and metabolites was achieved as described above, except that mobile phase A consisted of 5 mM ammonium acetate pH

4.5, the flow rate was 0.31 mL/min and the column size was 3.1 x 150mm, 3μm. MS/MS conditions consisted of declustering potential –26 V, focusing potential –350V, electrode potential –12V, cell entrance potential –16V, cell exit potential –20V and a temperature of 500°C. Identification of curcuminoids was by multiple reaction monitoring (MRM) using suitable transitions.

Pharmacokinetic analysis

Estimations of area under the plasma concentration curve (AUC) were obtained using a non-compartmental, extra-vascular plasma model with WinNonLin version 2.1.

Results and discussion

Rats received curcumin in unformulated or formulated form at a dose of 340 mg/Kg (in terms of curcumin) by oral gavage. Plasma, liver tissue and intestinal mucosa were obtained at several time points up to 2 hr post administration. Curcumin and species tentatively characterized as curcumin glucuronide, curcumin sulfate, tetrahydrocurcumin and hexahydrocurcumin were detected in plasma, intestinal mucosa and liver of rats after both administrations (Fig. 2 and 3). As the formulation used here has never been employed before, we wished initially to confirm the identity of detectable drug-derived species in animals, which had received formulated curcumin. To that end, extracts of bio-matrices were subjected to HPLC-mass spectrometric analysis. The analysis furnished incontrovertible proof of presence, based upon specific MRM transitions (in brackets), in the gut mucosa, plasma and liver of curcumin (367>134, Fig. 4 A) and of desmethoxycurcumin and bisdesmethoxycurcumin (337>119, Fig 4B and 307>119, Fig.4C), two curcuminoids co-extracted with curcumin from the *curcuma* plant and hexahydrocurcumin (373>179), curcumin sulfate (447>134) and desmethoxycurcumin sulfate (417>119, Figs. 4L, 4H and 4F). The curcumin metabolites, curcumin glucuronide (543>134) and tetrahydrocurcumin (371>135) were unambiguously identified only in gut mucosa and in plasma (Figs. 4D and 4I). Additional peaks were detected only in plasma, albeit at low abundance: glucuronides of desmethoxycurcumin (513>119), bisdesmethoxycurcumin (483>119), hexahydrocurcumin (549>179) and tetrahydrocurcumin (547>135, Fig. 4E, 4G, 4M and 4J, respectively). Sulfate metabolites of tetrahydrocurcumin (451>135) and hexahydrocurcumin (453>179) were detected in mucosa and in mucosa and liver respectively (Fig 4K and 4N).

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The occurrence of these species has previously been suggested in blood or tissues of rodents, which have received unformulated curcumin [7, 8], which suggests that formulation with phosphatidylcholine does not confound the qualitative pattern of curcumin metabolism *in vivo*.

Next, we compared plasma and tissue levels of curcumin in animals that had received either unformulated or formulated curcumin. Formulation dramatically and significantly increased curcumin levels in plasma (Fig. 5A) and liver (Fig. 6A) as compared to concentrations measured in animals that had received unformulated curcumin. Curcumin levels in the gut mucosa, observed after administration of formulated curcumin, were moderately lower than those after unformulated curcumin (Fig. 6B). These results clearly demonstrate that the administration of formulated curcumin is superior to that of unformulated curcumin if tissues other than the gastrointestinal tract are targeted, whilst maximal levels in the gastrointestinal tract can be achieved with unformulated curcumin. Both formulated and unformulated curcumin were completely removed from plasma within 2 h. Peak plasma levels of curcumin were approximately 5-fold higher for the formulated than for the unformulated agent (Table 1), although maximal systemic concentrations of curcumin achieved by administration of the formulated compound were still considerably below the values (>10-20 μM), which have been shown to elicit pharmacological effects in cells or cell free systems. Plasma levels of curcumin sulfate, curcumin glucuronide, tetrahydrocurcumin and hexahydrocurcumin observed after administration of formulated curcumin were 3- to 20-fold higher than those seen after unformulated curcumin (Fig. 5B, 5C, 5D, 5E and Table 1). Although too few data points were collected for robust pharmacokinetic analysis, tentative area under the curve (AUC) values were calculated from curcumin plasma concentrations. The plasma $AUC_{0-120\ min}$ for curcumin after administration of formulated curcumin was 5-fold higher than that for unformulated curcumin (Table 1). It is conceivable that the improved bioavailability of formulated curcumin increases the potential scope of medical applications for curcumin. Interestingly, in a recent study, intravascular administration of liposomally encapsulated curcumin using phosphocholine and phosphoglycerol technology was shown to suppress the growth of pancreatic tumours in nude mice [12]. These authors neither compared their formulation with unformulated curcumin, nor did they measure curcumin in the bio-matrix.

In conclusion, administration of curcumin formulated with phosphatidylcholine greatly increased plasma and hepatic bioavailability as compared to unformulated curcumin. Further investigation will show whether prolonged daily or multiple daily dosing of formulated curcumin is safe and can furnish tissue levels superior to those achieved with unformulated curcumin. The results presented here suggest that for chemoprevention intervention studies targeting sites other than the gastrointestinal tract, curcumin formulated with phosphatidylcholine may well be more advantageous than unformulated curcumin.

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Table 1 Estimated plasma Cmax, Tmax and AUC values for unformulated and formulated curcumin

	Cmax (nM)	T_{max} (min)	AUC (μg.min/mL)*
Unformulated			
Curcumin	6.5 ± 4.5	30	4.8
Curcumin glucuronide	225 ± 0.6	30	200.7
Curcumin sulfate	7.0 ± 11.5	60	15.5
Formulated			
Curcumin	33.4 ± 7.1	15	26.7
Curcumin glucuronide	4420 ± 292	30	4764.7
Curcumin sulfate	21.2 ± 3.9	60	24.8

* AUC was calculated using WinNonLin and employing a non-compartmental model.

Figure legends

Fig. 1 Structures of curcumin (A), desmethoxycurcumin (B), bisdesmethoxycurcumin (C), hexahydrocurcumin (D) and tetrahydrocurcumin (E).

Fig. 2 HPLC chromatograms of curcumin in 50% aqueous acetonitrile (A), an extract of rat plasma spiked with curcumin (B), or extracts of plasma (C), mucosa (D) or liver (E) from rats that received either curcumin (dotted line) or formulated curcumin (solid line). Bio-matrices were obtained 30 min post curcumin administration. In C, D and E solid lines and dotted lines represent bio-matrices from rats given formulated and non-formulated curcumin, respectively. Peaks 1-6 correspond to curcumin, desmethoxycurcumin, bisdesmethoxycurcumin, curcumin sulfate, curcumin glucuronide and desmethoxycurcumin glucuronide, respectively. For details of administration and HPLC analysis see Materials and methods.

Fig. 3. HPLC chromatograms of reduced curcumin metabolites tetrahydrocurcumin (open arrow) and hexahydrocurcumin (solid arrow) in 50% aqueous acetonitrile (A), extracts of rat plasma (B), mucosa (C) and liver (D). Bio-matrices were obtained 60 min post curcumin administration. In B, C and D solid lines and dotted lines represent bio-matrices from rats given formulated and non-formulated curcumin, respectively. For details of administration and HPLC analysis see Materials and methods.

Fig. 4 Representative LC/MS/MS chromatograms of extracts of plasma (A-J, L, and M) and mucosa (K and N) from rats, which had received formulated curcumin at 340 mg/Kg by oral gavage, demonstrating multiple reaction monitoring transitions indicative of curcumin (A), desmethoxycurcumin (B), bis-desmethoxycurcumin (C), curcumin mono-glucuronide (D), desmethoxycurcumin mono-glucuronide (E), desmethoxycurcumin mono-sulfate (F), bis- desmethoxycurcumin mono-sulfate (G), curcumin mono-sulfate (H), tetrahydrocurcumin (I), tetrahydrocurcumin mono-glucuronide J), tetrahydrocurcumin mono-sulfate (K), hexahydrocurcumin (L), hexahydrocurcumin mono-glucuronide (M), hexahydrocurcumin mono-sulfate (N). Tissues were obtained 30 min after curcumin administration. For details of administration and HPLC analysis see Materials and methods.

Fig. 5 Plasma levels of curcumin (A), curcumin glucuronide (B), curcumin sulfate (C), tetrahydrocurcumin (D) and hexahydrocurcumin (E) in rats, which had received curcumin (broken line) or formulated curcumin (solid line) at 340 mg/Kg by oral gavage. Values are the mean±SD (n = 3). Curcumin conjugated metabolite concentrations were estimated using the curcumin calibration curve. Star indicates that values at that time point were significantly different from each other ($p < 0.01$). For details of administration and hplc analysis see Materials and methods.

Fig. 6 Levels of curcumin in liver (A) and gastrointestinal mucosa (B) of mice, which had received curcumin (broken line) or formulated curcumin (solid line) at 340 mg/Kg by oral gavage. Values are the mean ± standard deviation (n =

3). Star indicates that values at that time point were significantly different from each other ($p < 0.01$). For details of administration and hplc analysis see Materials and methods.