Detection of plasma curcuminoids from dietary intake of turmeric-containing food in human volunteers.

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Short Running Title: PK analysis of curcumin from turmeric-containing foodstuffs.

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Abstract

Curcumin, (from turmeric), has been extensively investigated for potential beneficial properties in numerous diseases. Most work has focussed on supra-dietary concentrations/doses that would necessitate curcumin supplementation. However, much of the evidence instigating curcumin research is underpinned by epidemiological data based on low dietary intake via turmeric consumption.

To date, pharmacokinetics of curcumin from dietary intake of turmeric have not been reported due to lack of methodological sensitivity. Here, we describe a novel, highly sensitive LC-ESI-MS/MS method. Assay sensitivity was demonstrated in a pilot pharmacokinetic volunteer study following ingestion of foodstuffs containing a standardised mass of turmeric, representative of daily consumption by certain South Asian populations. Free parent curcumin was detectable in plasma from one individual, reaching a C_{max} of 3.2nM. Curcumin conjugates were detected in all volunteers; curcumin glucuronide achieved a maximum plasma concentration of 47.6±28.5nM 30 minutes post-food, whilst C_{max} for both demethoxycurcumin glucuronide and curcumin sulfate was ~2nM. Curcumin and its major metabolites persisted in plasma for at least 8 hours.

Despite poor absorption and rapid conjugation, dietary intake of turmeric furnished human plasma with detectable curcuminoids. Whether sustained low systemic concentrations over many years might have pharmacological activity for human health benefit, warrants further research.

Keywords: curcumin, turmeric, LC-ESI-MS/MS, healthy volunteer study

Introduction

There are many dietary-derived compounds with biological activity which have been studied for potential health benefit. One of the most extensively investigated of these is curcumin, which is contained in the spice turmeric. *In vitro* and *in vivo* studies have particularly alluded to its potential for anti-cancer effects, due largely to its pleiotropic nature and ability to target and disrupt multiple cellular signalling pathways implicated in the initiation and progression of cancer [1-3].

Whilst most clinical studies conducted to date focus specifically on the potential benefits of high dose curcuminoids for treatment of established disease, there has been less emphasis on disease prevention in healthy populations. Epidemiological evidence suggests a lower incidence of gastrointestinal cancers in those populations that coincidentally consume a turmeric-rich diet, which forms the source of curcumin [4-6]. Mechanistically, there is currently little evidence to suggest pharmacologic effect from the extremely low concentrations of curcuminoids that would arise from turmeric intake. However, this is partly because low dose effects have not been well studied and it is automatically assumed that 'more is better'. Conversely, it is increasingly being recognised that biological activity of dietary supplements may not always follow a linear dose-response relationship [7]. For example, recent evidence has demonstrated that the polyphenol resveratrol is more effective at preventing intestinal adenoma development in a mouse model and modulating certain signalling pathways at low dietary achievable doses compared to supra-dietary doses 200-fold higher [8].

It is now well established that curcumin undergoes rapid and effective first pass metabolism furnishing plasma with ng/mL levels of glucuronide and sulfate conjugate metabolites (Figure 1B), with free curcumin either undetected or found in negligible amounts following high-dose supplementation [9-11]. Clinical pharmacokinetic studies investigating curcumin have used doses ranging from 0.4 g to 12 g/day but most have failed to detect free curcumin, particularly at the lower doses, unless strategies have been employed to enhance bioavailability, such as micellar, phospholipid or nanoparticulate formulations, or co-administration of adjuvants that can block key metabolic pathways [12-15]. For this reason, studies that attempt to investigate the

biological effects of curcumin from dietary-achievable doses of turmeric *in vivo* are hindered, as it cannot be proven that curcumin/curcuminoids are systemically available.

In order to further establish whether consumption of turmeric generates detectable systemic levels of curcuminoids in plasma, we developed a new sensitive LC-ESI-MS/MS method and undertook a pilot pharmacokinetic study in healthy volunteers who had ingested known quantities of turmeric (~3 g) within a food matrix, equivalent to a total curcumin intake of ~100mg. Dietary curcumin intake has been estimated in European populations, with a study in France suggesting consumption of 0.48 mg/day/kg body weight for curcumin in the form of food additive E100 [16]. In South Asia where turmeric is consumed more regularly, the estimated average daily intake of turmeric is 1-2 g per person [17], and this translates to 80 – 200 mg of curcumin per day [18]. Therefore, the quantity of turmeric used in the present study (3 g) represents delivery of a feasible dietary curcuminoid dose.

Here, we describe a novel, partially validated assay for detection of low plasma concentrations of curcuminoids derived from a dietary-feasible intake of turmeric, and demonstrate the clinical utility of the method through application to a human volunteer study.

Material and Methods

Chemicals and Reagents

Standard curcumin was obtained from Indena SpA. (Milan, Italy). Dimethylsulfoxide (DMSO) was purchased from Sigma Aldrich (Dorset, UK). Curcumin glucuronide and sulfate standards were synthesized as the mono-substituted metabolites in our laboratory using methods described by [19] and [11], respectively. The standards were characterised by mass spectrometry (MS) and nuclear magnetic resonance (^{1}H-NMR). The purity of the curcumin sulfate standard was ≥97% and that of curcumin glucuronide was ≥95%. HPLC/LC-MS grade acetonitrile, acetone, formic acid and acetic acid were purchased from Fisher Scientific Ltd. (Loughborough, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Ltd. (Hamspshire, UK). LC-MS optima grade water was obtained from Fisher Scientific

Ltd (Loughborough, UK), and ultrapure water (18.2 MΩ quality) was obtained from a Nano-pure water purification system (Barnstead, UK).

Food Preparation

This study was undertaken to investigate the pharmacokinetics of curcumin when taken in the form of turmeric present in cooked food. The food used for the study consisted of two savoury items, a sandwich made from turmeric-containing bread and a portion of soup, plus a sweet oat bar (flapjack) (breakdown for nutritive constituents shown in supplementary table 2, and further supplementary information). All food was prepared by the University of Leicester Catering Department, such that each portion contained 1 gram of turmeric (Figure 1A); each participant therefore consumed 3 g turmeric in total equating to approximately 100 mg of curcumin, given that the proportion of curcumin in the batch of turmeric used for cooking was 3.25%. The variety of food represents that which could be readily consumed as part of a 3-course meal. Three grams of turmeric as a single dose in food produces a strong taste and grainy texture, hence the turmeric dose was split between 3 food items, to produce a palatable menu. Acceptability of a range of menu items containing 1 g turmeric were first tested and rated by University staff members, prior to the most successful recipes being utilised in this study and subsequently going on sale throughout University catering outlets. The curcumin content of the ingredient turmeric powder and samples of the three food items was determined using a previously established and validated HPLC-UV method developed in our laboratory [20].

Study Design

Four volunteers aged 26-35 years (3 male and 1 female) were recruited into the study following approval from the University of Leicester local ethics committee (reference lh28-3072). All volunteers were without any known relevant medical conditions (including allergies or food intolerance) and were not on any medication. Biochemical measures of health were not undertaken at baseline, meaning that self-reported health status could not be confirmed. All volunteers gave written informed consent for the study. The volunteers were asked to refrain from all turmeric-containing food or drink for the 24 h preceding and during the study, and were required to fast for 12 h prior to study commencement. After the first blood draw at 0

h, each volunteer consumed three portions of turmeric-containing food consisting of soup, a sandwich and a flapjack. The subsequent blood draws were at 30 min, 1, 2, 4, 8 and 24 h post food consumption. Other than refraining from turmeric-containing food, subsequent food intake was not restricted; participants were free to eat whatever they chose. Blood samples were collected in heparinised tubes and centrifuged at 3300 *x g* for 10 min at 4ºC. Plasma was separated and aliquoted into 1 mL volumes then stored at -80ºC until further use. All blood samples were processed within 30 min from the time of collection.

Sample extraction procedure

To a 250 µL aliquot of each plasma sample, 500 µL of acetone/formic acid (9:1, *v/v*) was added, vortexed for 10 s and then placed at -20°C for 30 min. The mixture was then centrifuged at 17,000 x *g* for 20 min (4°C) and the supernatant transferred to a fresh eppendorf tube. The supernatant was concentrated to dryness using a SPD100 SpeedVac centrifugal evaporator, the dried residue re-suspended in 50 µL of 0.1% acetic acid/acetonitrile (0.1% acetic acid) (40:60 *v/v*), vortexed for 10 s and then centrifuged at 17,000 x *g* for 3 min. Supernatant was transferred to HPLC vials and a 20 µL aliquot injected onto the LC-ESI-MS/MS system.

LC-ESI-MS/MS analysis of curcumin and metabolites

The LC-ESI-MS/MS system consisted of a Waters Alliance 2695 separation module with a 100 µL injection loop and Waters 2487 UV detector connected to a Micromass Quattro Platinum (Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 120ºC and the desolvation temperature at 350ºC. Nitrogen gas was used as the desolvation gas (650 L/h), the cone gas was set to 25 L/h, and capillary voltage set to 2.85 kV. The cone and RF lens 1 voltages were 45 V and 30 V, respectively, with aperture and RF lens 2 voltages being 0.4 V and 1.0 V, respectively. The mass spectrometer was tuned by using curcumin and curcumin glucuronide and sulfate metabolite standard solutions (50 pmol/µL) dissolved in 0.1% acetic acid/acetonitrile (0.1% acetic acid) (40:60 *v/v*) introduced by continuous infusion at a flow rate of 5 µL/min with a Harvard model 22 syringe pump (Harvard Apparatus Ltd., Edenbridge, UK).

A 20 µL aliquot of sample was injected onto a HyPurity C18 (2.1 × 150 mm, 3 µm) column connected to a HyPurity C18 (2.1 × 10 mm, 3 µm) guard cartridge (Thermo Electron Corporation, Runcorn, UK) attached to a KrudKatcher (5 µm) disposable pre-column filter. The column was eluted using a gradient with solvent A, 0.1% acetic acid and solvent B, acetonitrile (0.1% acetic acid) at a flow rate of 200 µL/min and a run time of 45 min. The following gradient was used: 0min-10%B, 15min-40%B, 25min-85%B, 30min-100%B, 35min 100%B, 35.1min-10%B and 45min-10%B. The column oven temperature was maintained at 30 ºC, with the UV detector operated in dual absorbance mode monitoring at 280 and 426 nm.

The samples were analysed in negative electrospray ionization (ESI) mode with selected reaction monitoring (SRM) for the $[M-H]^-$ ion transitions of curcumin and metabolites: curcumin glucuronide 543 to 217 *m/z*, demethoxycurcumin glucuronide 513 to 119 m/z, curcumin sulfate 447 to 217 *m/z*, and curcumin 367 to 149 *m/z*. The collision gas was argon (indicated cell pressure 2.5×10^{-3} mbar) and the collision energy set to 18 eV. The dwell time was set to 100 ms and the resolution 1.0 *m/z* units at peak base.

Data Acquisition

The data were acquired using MassLynx software (Version 4.0). The levels of curcumin and the metabolites in the samples were determined from individual calibration lines constructed by addition of different amounts of curcumin, curcumin glucuronide and curcumin sulfate standards ranging from 20 to 1000 fmol, or for curcumin glucuronide, ranging from 20 to 20,000 fmol detected on column, to the control sample matrix.

Results and Discussion

Assessing metabolite profiles generated by consumption of food containing active constituents such as curcuminoids presents many challenges. The bioavailability of biologically active compounds may be altered when present within complex food matrices, cooking processes may chemically change the nature and amount of the parent compound, and intake at a dietary dose may generate such low systemic levels that they are extremely difficult to detect in human samples. To this end, we sought to develop an LC-ESI-MS/MS method that would be sensitive enough for

detection of plasma curcuminoids after administration of turmeric-rich foodstuffs to healthy volunteers.

The newly developed LC-ESI-MS/MS method was first partially validated using FDA draft guidance for bioanalytical method validation [21], prior to use for the analysis of human samples from the intervention study. Partial validation can refer to a wide range of criteria, and so assay specifications and the degree to which the method was validated, are presented below.

Accuracy and Precision

Accuracy and precision were determined by replicate analysis (n=3) of control blank plasma spiked with 25, 250 or 1000 fmol of the curcumin, curcumin glucuronide and curcumin sulfate standards, reflecting expected study sample concentration range. Table 1 summarises intra-day and inter-day precision and accuracy for the spiked plasma samples. The criterion of mean values within 15% for accuracy and coefficient of variance (CV) not exceeding 15% for precision, was achieved as per guidance specifications [21].

Selectivity

The selectivity of the method was assessed by comparing LC-MS/MS SRM chromatograms obtained for control blank extracted human plasma with that of extracted human plasma spiked with 1000 fmol of each analyte. There was no interference due to the presence of any endogenous peaks or impurities present in control blank extracted plasma within the retention time region of interest for all three analytes (Figure 2). This also held true for the lower limit of quantitation (LLOQ), although the matrix sample was from one source only rather than from 6 separate sources as per recommendations for full validation.

Calibration Curve

Calibration curves for all the three analytes were obtained by spiking the analytes at six concentrations over the range 20-1000 fmol for the curcumin and curcumin sulfate standards, and 20-20,000 fmol for the curcumin glucuronide standard in control blank extracted human plasma matrix, in keeping with expected ranges. The relation between concentration and response was continuous and reproducible. The individual correlation coefficients (r^2) for each calibration line were 0.999, 0.999 and 0.998 for curcumin, curcumin sulfate and curcumin glucuronide respectively, and did

not deviate by more than 15% of nominal concentrations, with >75% of non-zero standards conforming to this as per FDA guidance.

Limit of detection

The limit of detection (LOD) for the LC-MS/MS method was calculated as the lowest amount of analyte on column required to produce a signal to noise (S/N) ratio of 3. The limit of detection values were 2.5 (0.125 nM), 10 (0.5 nM) and 5 (0.25 nM) fmol detected on column for curcumin, curcumin glucuronide and curcumin sulfate respectively. LLOQ values were 20 (1 nM), 20 (1 nM) and 50 (2.5 nM) fmol detected on column for curcumin, curcumin glucuronide and curcumin sulfate, respectively. The upper limit of quantitation (ULOQ) was not defined due to limited supply of sulfate and glucuronide standards.

Recovery

The recovery was assessed by comparing peak areas detected from spiked and extracted plasma samples with peak areas produced by direct injection of a known quantity of each standard. The recovery values for the three analytes were calculated for 1000 fmol spiked amounts. The percentage recovery values were 76.5±4.6, 68.0±11.1 and 75.0±16.3 for curcumin, curcumin glucuronide and curcumin sulfate respectively, obtained across 6 replicate determinations.

For full validation, the method would benefit from use of stable-isotope-labelled standards for each analyte, which are now available from commercial sources. Determination of upper limits of quantitation (ULOQ) in addition to freeze-thaw stability experiments would be extra requirements to complete full validation. However, previous experience with curcuminoid stability revealed that curcumin, and its glucuronide and sulfate conjugates were stable in the autosampler and for up to 3 freeze-thaw cycles. Long-term storage (up to 3 months) resulted in loss of all 3 species in plasma, with the most substantial loss observed for curcumin sulfate (up to 23%) [22]. This compares to a more recent study which suggested only an 11% loss of the sulfate following 2 months long-term storage at -70°C [23].

Determination of curcumin content in food items

Prior to analysing the human plasma samples, it was imperative to determine the curcumin content of the ingredient turmeric powder and in the three food items used in the study. Employing a previously established and validated HPLC-UV method [20] the curcumin content of turmeric was found to be 3.25% (approximately 4.25% total curcuminoids), which is consistent with reported concentrations in commercially available turmeric [24] (Figure 1C). The curcumin content was 71, 52 and 48% of the expected levels in soup (which was freeze-dried before extracting), bread and the oat bar, respectively, based on each portion and food type containing 1 g of turmeric prior to being cooked. The reason these values are less than 100% can be attributed to a variety of factors, the contribution of which may differ across the food items. Losses due to curcumin degradation/chemical modification during the cooking procedure are likely to be a significant issue, along with incomplete extraction from complex food matrices. The effect of such matrices on polyphenol accessibility from the diet is well established. Foods that are high in fibre or protein-rich may decrease polyphenol accessibility whereas those with a high lipid content (particularly relevant for hydrophobic molecules such as curcumin) may increase availability. Ultimately, polyphenols are reliant on their release from food via favourable conditions produced by the gastric phase of the digestive process[25]. It is worth noting that since the extraction methods were not designed to simulate the gastrointestinal environment, the values may not be an accurate representation of the true availability of curcuminoids in humans after oral ingestion, and could even be an underestimation. Curcuminoids also lack stability at neutral or alkaline pH and in aqueous solution [26], and can be subject to bacterial degradation [27]. Additionally, extraction from complex matrices may contribute to analyte signal suppression in ESI due to co-elution of molecules, followed by their ionization in the liquid phase before being released in the gas phase [28].

Nevertheless, the results show that a good proportion (at least 48 - 71%) of the added curcumin survived the cooking process and was theoretically available for absorption after ingestion. Apart from the curcuminoid peaks, no other peak could be detected by HPLC-UV at 426 or 280nm.

Volunteer study PK analysis

Plasma samples from volunteers were analysed using the newly validated ESI LC-MS/MS method. In plasma samples taken before food consumption, there was no detectable curcumin or sulfate/glucuronide conjugates in any of the volunteers (Figure 3A1-D1). Following food consumption, parent curcumin was detected in only one out of four volunteers with a maximum concentration (C_{max}) of 3.2 nM achieved 2h post food ingestion (Figure 3, Table 2). Major metabolites of curcumin including curcumin glucuronide, demethoxycurcumin glucuronide and curcumin sulfate were detected in all 4 volunteers. Figure 4 represents the average plasma concentration *vs* time curves for all the detected analytes across the four volunteers and Table 2 summarises the corresponding C_{max}, T_{max} and T_{last} values. The demethoxycurcumin glucuronide concentration was estimated using the curcumin glucuronide calibration line, as an authentic standard was not available for this compound.

Curcumin glucuronide was found to be the most abundant metabolite; it was first detected 30 min following food consumption and generated an average C_{max} of 47.6±28.5 nM, which was achieved 30 min post food. The concentration decreased after this point in a time-dependent manner but circulating glucuronide could still be detected at 24 h, at concentrations of ~0.2 nM in two out of the four volunteers. Based on the mean data, the last time point at which curcumin glucuronide could be detected at a concentration above the LOD (T_{last}) was 24h.

For curcumin sulfate, the average C_{max} was 2.1±1.7 nM, achieved at 30 min post food consumption. The concentration then declined rapidly and the metabolite was completely eliminated by all four individuals before 24h. In addition to the predicted major conjugated curcumin metabolites, demethoxycurcumin glucuronide was also detected in plasma samples from all four volunteers with an estimated average C_{max} value of 1.9±1.2 nM at 30 min, which is ~25-fold lower than the concentration of mono-glucuronidated curcumin.

There is evidence to suggest that gender can have a significant influence on curcumin pharmacokinetics[15], with C_{max} plasma levels following administration of an oral micellar curcumin formulation to healthy human volunteers, reportedly 1.4-fold higher in females versus males. The authors hypothesised that these differences may be due to gender-specific factors including increased activity of hepatic drug efflux transporters in males, and higher body fat in females, effectively increasing the

dose per kg body weight in females. It is also important to consider that the administered dose per kg of body weight likely differs between genders, and so may have contributed to observed differences in plasma concentrations. The study presented here involved only 1 female participant, in whom C_{max} plasma levels of the glucuronide, DMC glucuronide, and sulfate were all lower than in the male study participants (Supplementary Table 1). In addition, parent curcumin was detected only in 1 of the male volunteers. We acknowledge that numbers are too small to add to any previous observations of curcumin uptake between genders. However, the primary aim of this study was as proof of principle that the method was sufficiently sensitive to detect curcuminoids from a feasible dietary turmeric intake.

The dose given was similar to the average daily turmeric consumption in India, which can be as high as 2–2.5 g per day (again corresponding to up to ~100 mg of curcumin) [29]. Analysis of the food items used in the study demonstrated that at least ~50% of the added curcumin survived the various cooking processes. Pyrolysis of curcumin at high temperatures with and without coconut fat or olive oil, has been shown to cause degradation, leading to more polar products including deketene curcumin which has been reported to have cytotoxic activity in mouse melanoma cells superior to curcumin itself [30]. We chemically synthesized a standard of this degradation product but found no evidence of its presence in the food samples using a HPLC-UV method [31] with detection at both 426 and 280 nm (data not shown). However, the methods were not optimized for this particular derivative and we did not search for other potential degradation products of curcumin, so it is conceivable that they were present in low amounts but were not detected using our assay. Alternatively, deketene curcumin may not have been formed at the temperatures involved in cooking the soup, bread and oat bar.

In the current study, we used an extremely low dose of curcumin (equating to approximately 1.5 mg/kg for a 70 kg adult) in the form of food preparations to investigate whether curcumin and/or its metabolites could be detected after ingestion of a dietary-achievable quantity within a food matrix. Whilst many investigations (both clinical trials and healthy volunteer studies) have used supra-dietary doses of

standard curcumin [13, 32], very low doses have also been administered (~ 10 – 400 mg), often in formulations designed to enhance bioavailability or in combination with adjuvants, which have afforded much greater systemic concentrations [13, 33, 34]. However, Sharma et al [35] in early work within our group, investigated the pharmacokinetics of curcumin (not formulated for greater bioavailability) at doses encompassing 36 – 180 mg of daily oral curcumin. Here, curcuminoids could not be detected in plasma samples either directly, or following enzymatic conversion using β-glucuronidase/sulfatase to hydrolyse curcumin conjugates. Curcumin levels at doses of > 144 mg, furnished only fecal matter with detectable levels of curcumin. The inability to detect plasma curcuminoids in this early study was likely due to poor methodological sensitivity which has now been resolved in the study presented here. Sasaki et al [36] assessed comparative pharmacokinetics between curcumin (30 mg/kg body weight, equating to approximately 210 mg for a 70 kg individual) and the Theracumin formulation. Here, a curcumin C_{max} of 4.89 nM was reported following standard curcumin dosing (which is in keeping with curcumin levels found in our volunteer study), but the major conjugates were not evaluated despite their potential for efficacy. In the majority of the low dose studies assessing curcumin formulated specifically to enhance bioavailability, the major conjugate metabolites were similarly not assessed, and detection of parent curcuminoids was undertaken only via hydrolysis of plasma curcumin conjugates. Summaries for a selection of low dose studies are shown in supplementary table 3 [35-46].

The method described here, successfully detected and quantitated conjugated metabolites, with free curcumin detected in one out of the four volunteers. These results provide proof of principle that free curcumin reaches the systemic circulation after dietary consumption of turmeric-containing foods. A recent clinical pharmacokinetic study used a single oral dose of 2 g curcuminoids taken as a nanoemulsion formulation, and quantified concentrations using an LC-MS/MS method [47]. In their study, curcumin reached a C_{max} of 0.03 μM, whilst the mono-glucuronide and sulfate metabolites afforded maximum concentrations of 1.8 μM and 0.3 μM, respectively. These levels are 38 - 140-fold higher than the concentrations generated in the present study, which reflects the 20-fold difference in curcumin intake, and likely discrepancies in absorption from food versus pharmaceutical formulations. Whilst other validated LC-MS/MS methods have been developed for

the detection of curcuminoids [48] [23, 49], some of which report LODs in the region of 2-7 nM for curcumin and its metabolites [47] [50], curcumin pharmacokinetics from food intake have not previously been assessed.

The plasma concentrations of curcumin generated through dietary consumption of turmeric are far lower than those routinely shown to elicit biological responses using preclinical *in vitro* models, and also the comparable doses employed for *in vivo* assessment of efficacy, across a variety of pathologies[2, 51, 52]. However, such low concentrations have not typically been studied and it is possible that with long term repeated exposure they may have some activity. Another possibility, for which there is emerging evidence, is that curcumin metabolites, which attain much higher concentrations, may play an important role in mediating activity. Whilst we have previously shown that the major curcumin metabolites are less efficacious at inhibiting prostaglandin E2 (PGE2) production compared to parent curcumin [53], comparisons were made at equivocal concentrations and so did not reflect the 10-fold differences in concentrations that can arise in humans. An *in vivo* study has demonstrated anti-cancer effects of curcumin glucuronide when injected intravenously into HCT116 human colon tumour bearing mice [54]. The same study also showed that free curcumin is generated in the plasma of these animals after IV administration of the glucuronide. This back conversion is probably due to the endogenous activity of glucuronidase and sulfatase enzymes expressed in different organs, including the gastrointestinal tract. Other curcumin metabolites such as tetra- and hexahydrocurcumin (which were not seen in this study) are also thought to offer anti-cancer efficacy *in vivo* [55, 56]. It is therefore important to consider evaluation of the major conjugates when using standard curcumin or curcumin from a native source such as turmeric.

Conclusion

Most curcumin research to date has focussed on supra-dietary doses, and ways to increase curcuminoid bioavailability. Here, we have developed and utilised a new, sensitive, partially validated LC-ESI-MS/MS method. This is the first study to show that low nanomolar concentrations of curcumin and its metabolites following a feasible turmeric intake from dietary sources, are readily detectable in human

subjects, with free parent curcumin also detectable in one out of four volunteers. The question of whether such low systemic levels sustained over many years through dietary intake have pharmacological activity that translates to human health benefits, warrants further research.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends:

Figure 1. (A) Examples of the foods consumed by the volunteers in this study. (**B**) Major curcumin metabolites in humans following oral administation. (**C**) Representative HPLC-UV chromatograms from the analysis of turmeric and turmeric containing food used for the healthy volunteer study. Each portion of food contained 1 g of turmeric. Peaks 1-3 correspond to: (1) curcumin, (2) demethoxycurcumin, (3) bis-demethoxycurcumin.

Figure 2. LC-MS/MS SRM chromatograms for transitions corresponding to curcumin glucuronide (543 > 217 *m/z*, **A1, A2**), curcumin sulfate (447 > 217 *m/z*, **B1, B2**) and curcumin (367 > 149 *m/z*, **C1, C2**) in control blank human plasma (**A1**-**C1**) and human plasma spiked with 1000 fmol of each analyte (**A2**-**C2**). The retention times of curcumin glucuronide, curcumin sulfate and curcumin were 20.9, 24.6, 25.8 min, respectively.

Figure 3. Representative analysis of plasma samples from a volunteer pre and post food consumption. LC-MS/MS SRM chromatograms for transitions corresponding to curcumin glucuronide (543 > 217 *m/z*, **A**1, **A2**), demethoxycurcumin glucuronide (513 > 119 *m/z*, **B1**, **B2**), curcumin sulfate (447 > 217 *m/z*, **C1**, **C2**) and curcumin (367 > 149 *m/z*, **D1**, **D2**) in human plasma. The left hand panel (**A1**-**D1**) corresponds to a plasma sample collected before food consumption. The right hand panel (**A2**-**D2**) corresponds to a plasma sample from the same human volunteer collected 30 min following food consumption. The retention times of curcumin glucuronide, demethoxycurcumin glucuronide, curcumin sulfate and curcumin were 21.3, 20.9, 24.7, 26.4 min, respectively. The extra two peaks present in the curcumin SRM channel correspond to the generation of curcumin from the source-induced dissociation of curcumin glucuronide and curcumin sulfate.

Figure 4. Average plasma concentration *vs* time profile of (**A**) curcumin, (**B**) curcumin glucuronide, (**C**) demethoxycurcumin glucuronide[a] and (**D**) curcumin sulfate in healthy human volunteers after consumption of food containing approximately 3 g of turmeric. Values are presented as mean ± standard deviation (n

= 4), apart from the data for curcumin (**A**), since this was only detected in a single human volunteer.

[a] The demethoxycurcumin glucuronide concentration was estimated using the curcumin glucuronide calibration line as an authentic standard was not available for this compound.

Table legends:

Table 1. The accuracy and precision of the method for the detection of curcumin, curcumin glucuronide and curcumin sulfate in blank human plasma matrix.

Table 2. Average curcumin and curcumin metabolite concentrations in the plasma of healthy volunteers after administration of food containing approximately 3 g of turmeric.

Supplementary Table 1. Curcuminoid C_{max} by gender.

Supplementary Table 2. Fat, protein and carbohydrate content of each food type (per 100 g).

Supplementary Table 3. Summary characteristics of some of the low dose studies of curcumin in which a dose <500 mg was used.

Further supplementary information - Turmeric recipes with nutrition.