An HPLC-UV method for the simultaneous quantification of curcumin and its metabolites in plasma and lung tissue: potential for preclinical applications.

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Short Running Title: Quantification of curcumin and its metabolites in plasma and lung.

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Abstract

Curcumin, derived from turmeric, has been extensively investigated for its broad spectrum of biological activities. Previously reported HPLC-UV methods have focussed on analysis of the parent compound. Here, a sensitive HPLC-UV method was developed and partially validated, then used for the simultaneous determination of curcumin and its glucuronide and sulfate metabolites in plasma and lung tissue from mice. The assay was applied to an *in vivo* pharmacokinetic study comparing formulated curcumin (Meriva™) with standard curcumin. Plasma levels of glucuronide and sulfate metabolites were 5-fold and 2-fold higher after Meriva™ administration compared to standard curcumin. In lung tissue, free curcumin was 4-fold higher following Meriva™ administration versus standard curcumin. This assay represents a rapid, cheap method for simultaneous detection of curcumin and its major metabolites that has applicability in pre-clinical settings.

Keywords: Curcumin; Curcumin Sulfate; Curcumin Glucuronide; HPLC-UV; Lung Cancer

1.0 Introduction

Turmeric, derived from the roots of the *curcuma longa* plant, has traditionally been used as a dietary constituent and herbal remedy for a variety of ailments and diseases. The major pharmacologically active constituent of turmeric, constituting approximately 3-4%, is curcumin [(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. Curcumin exhibits a wide range of biological activities including anti-cancer (Aggarwal et al. (2003); (Howells et al., 2011; James et al., 2015), anti-oxidant (Ak and Gulcin, 2008), anti-inflammatory (Jurenka, 2009), anti-bacterial (Moghadamtousi et al., 2014; Teow et al., 2016) and anti-diabetic effects (Zhang et al., 2013) in numerous *in vitro* and *in vivo* studies. Recent reports have also suggested its potential for use against other diseases including HIV (Guo et al., 2013), Alzheimer's (Ahmed and Gilani, 2014), cardiovascular conditions (Wongcharoen and Phrommintikul, 2009; Kapakos et al., 2012) and respiratory diseases (Moghaddam et al., 2009). Curcumin owes its biological properties, at least in part to its molecular structural functionalities, i.e. the β -diketone moiety and the phenolic hydroxyl and methoxy groups, which probably mediate its interaction with various target proteins (Gupta et al., 2011). Commercially available curcumin preparations typically contain approximately 80% curcumin, 17% demethoxycurcumin (DMC) and 3% bis-demethoxycurcumin (bDMC), which collectively are referred to as curcuminoids (Supplemental Figure S1).

Pharmacokinetic studies of curcumin undertaken in rodents or humans have shown that curcumin has very low bioavailability. Following an oral dose of 2 g in humans, curcumin was either undetectable or detected at very low levels (0.006 ± 0.005 $\mu\text{g/mL}$) in the serum, with an equivalent dose in rats producing a maximal concentration of 1.35 ± 0.23 $\mu\text{g/mL}$ (Shoba et al., 1998). Poor aqueous solubility, leading to inefficient absorption and rapid first pass metabolism are considered to be the primary reasons for the low bioavailability of curcumin. Following oral administration, curcumin is rapidly converted into glucuronide and sulfate metabolites. On intravenous or intraperitoneal injection, curcumin is also reduced to dihydrocurcumin (DHC), tetrahydrocurcumin (THC) and hexahydrocurcumin (HHC), of which THC is the predominant species. Other metabolites of curcumin are curcumin glucuronide-sulfate and glucuronide conjugates of THC and HHC (Anand et al., 2008).

A number of analytical methods have been described for the detection of curcumin, amongst which, high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection is the most commonly

used (Heath et al., 2003; Pak et al., 2003; Ma et al., 2007; Li et al., 2009). However, most of these methods focus on the parent molecule and rarely consider metabolite recovery, stability or specificity. There is tentative evidence suggesting that curcumin may be beneficial in terms of delaying the development of lung cancer in a limited number of animal models (Howells et al., 2014). In order to study the pharmacokinetics and pharmacodynamics of curcumin in the lungs, a robust analytical method is required which allows consistent, reliable and accurate detection of curcumin and its metabolites not only in the plasma but also in lung tissue.

Here, we describe a HPLC-UV method for the simultaneous quantitation of curcumin and its major metabolites curcumin glucuronide and curcumin sulfate in plasma and lung tissue from mouse biomatrices. Further, we have conducted a comparative oral bioavailability study between a curcumin phospholipid formulation (Meriva™) and standard curcumin to examine levels achieved in mouse plasma and lungs, at a low dose which equates to approximately 400 mg in human adults.

The ability to accurately detect and quantify curcuminoids at concentrations generated by doses which are relevant to those being explored within the clinical arena, is crucial for assessing pharmacokinetics, disposition and compliance monitoring in clinical trials of curcumin, with particular emphasis on lung cancer chemoprevention. This is the first study that describes a **partially-validated** method capable of simultaneously detecting curcumin and the major curcuminoid metabolites in both plasma and lung tissues. In addition, this method has been successfully translated to a low dose murine pharmacokinetic study.

2.0 Material and Methods

2.1 Chemicals and Reagents

Standard curcumin and Meriva™ were obtained from Indena SpA. (Milan, Italy). Ammonium acetate, dimethylsulfoxide (DMSO) and β -glucuronidase from *Helix Pomatia* were purchased from Sigma Aldrich (Dorset, UK). The β -glucuronidase enzyme also contained sulfatase activity. Curcumin glucuronide and curcumin sulfate standards were synthesized as the mono-substituted metabolites as described by Pal *et al.* (Pal et al., 2014) and Irving *et al.* (Irving et al., 2013), respectively. The standards were characterised by mass spectrometry (MS) and nuclear magnetic resonance ($^1\text{H-NMR}$). The purity of curcumin sulfate standard was $\geq 97\%$ and that of curcumin glucuronide was $\geq 95\%$. HPLC grade acetonitrile, acetone and acetic acid were purchased from Fisher Scientific Ltd. (Loughborough, UK). Phosphate buffered saline (PBS) tablets were

purchased from Oxoid Ltd. (Hampshire, UK). Water used in the analyses was obtained from a Nano-pure water purification system (Barnstead, UK).

2.2 Sample collection

Mouse blood and lung tissue were obtained under project license PPL40/2496 and 60/4370, granted to the University of Leicester by the United Kingdom Home Office, vetted by Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCR for animal welfare (Workman et al., 2010). Blood was collected into heparinised tubes (Startstedt AG and Co., Nümbrecht, Germany) and plasma was separated by centrifugation at 16200 x g for 20 min at 4°C. The plasma was then transferred into new eppendorf tubes and stored at -80°C until further use. Lung tissue was snap frozen in liquid nitrogen at the time of harvesting and then homogenised in PBS at the ratio of 1:2 w/v with an X-1020 tissue homogenizer (Ystral GmbH, Döttingen, Germany) and stored at -80°C.

2.3 Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of curcumin, curcumin glucuronide and curcumin sulfate were prepared at a concentration of 1 mg/mL in DMSO. These solutions were further diluted to give final concentrations ranging from 0.05 - 5 µg/mL. All stock solutions were prepared in amber coloured eppendorfs and stored at -20°C for not more than one month after preparation. Calibration standards were prepared by spiking curcumin, curcumin glucuronide and curcumin sulfate together in plasma or lung homogenate. One µL from the stock solution of each analyte was added to 100 µL of plasma/lung homogenate, in order to obtain a calibration curve spanning 7 concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, 5 µg/mL) for each analyte. Quality control (QC) samples were similarly prepared at the following concentrations: 0.1 (low), 2 (medium) and 5 µg/mL (high) for plasma and 0.5 (low), 2 (medium) and 5 µg/mL (high) for lung tissue, and stored at -80°C prior to use.

2.4 Sample preparation

Formic acid:acetone (1:9, v/v, 200 µL) was added to 100 µL of spiked plasma or lung homogenate, vortexed for 10 seconds and kept at -20°C for 30 min. The mixture was centrifuged (13000 x g) for 20 min at 4°C, supernatant transferred to a fresh eppendorf tube, and the precipitate re-extracted. The supernatant from the two extractions was pooled together and evaporated to dryness using a SPD100 SpeedVac® System (Thermo

Scientific, UK) with no heat. The dried residue was re-suspended in 10 mM ammonium acetate (100 µL, pH 4.5):acetonitrile, (50:50, v/v) vortexed, centrifuged at 16200 x g for 3 min and the supernatant transferred to HPLC vials.

2.5 HPLC-UV instrumentation and chromatography parameters

The HPLC-UV system consisted of a Waters Alliance 2695 system with a built-in quaternary gradient proportioning valve, degasser and autosampler, coupled with a Waters 2487 Dual λ UV/VIS absorbance detector. The detection wavelength for curcumin, curcumin glucuronide and curcumin sulfate was 426 nm. Curcumin and its metabolites were separated using an Atlantis® dC18 reverse phase-HPLC column (4.6 x 150 mm, particle size 3 µm, Waters, Hertfordshire, UK) using gradient elution and an Atlantis® dC18 guard column (4.6 x 20 mm, particle size 3 µm, Waters, Hertfordshire, UK) maintained at 25°C. The two mobile phases consisted of 10 mM ammonium acetate, pH 4.5 (mobile phase A), and acetonitrile (mobile phase B). Mobile phase A commenced at 90%, progressing to 60% at 15 min, 15% at 25 min, 0% at 30 min, and to 90% at 30.1 min until the end of the run (40 min). Injection volume was 50 µL, with flow rate set at 1 mL/min, and auto sampler temperature at 4°C. Data acquisition was via Empower software.

2.6 Method Validation

Validation was performed in accordance with FDA bioanalytical method validation guidelines for industry (FDA, 2001).

2.7 *In vivo* pharmacokinetic study

C57 BL/6J male mice 6 weeks of age (Charles River Laboratories, UK) were kept under a 12 h light/dark cycle on standard AIN93G diet. Animals (three per time point) were fasted overnight and received Meriva™ or standard curcumin at a dose of 70 mg/kg in terms of curcumin equivalent, by oral gavage. Blood samples were obtained under anaesthetic by cardiac puncture at 0.25, 0.5, 1, 1.5 and 3 h post dose, and plasma derived by centrifugation at 13000 x g for 20 min. Plasma was stored at -80°C until analysis. Following cervical dislocation, lung tissues were harvested, snap frozen, and stored at -80°C until analysis. Plasma and lung samples were analysed by HPLC-UV as described above.

2.8 Conversion of curcumin metabolites to parent curcuminoids

Phosphate buffer (0.1 M, pH6.8) was used to dissolve β -glucuronidase/sulfatase to give 1800 units of β -glucuronidase and 160 units of sulfatase per 100 μ L. β -glucuronidase/sulfatase solution (100 μ L) was then added to 100 μ L of plasma/lung homogenate, vortexed vigorously and incubated at 37°C for 3.5 h, prior to extraction and subsequent analysis.

3.0 Results and Discussion

3.1 Selectivity and sensitivity

Selectivity of the assay was studied in murine plasma and lung tissue. No components were detected in blank plasma and lung tissue samples which could have interfered with the detection of curcuminoids, curcumin glucuronide or curcumin sulfate (Figures 1A and B). Representative chromatograms of plasma and lung samples spiked with the standards (Figures 1C and D) demonstrated that all the peaks detected were well resolved from each other. When this method was applied to an *in vivo* pharmacokinetic study, similar resolution was obtained for curcumin species extracted from plasma (Figure 1E) with no other endogenous peaks detected. No peaks were detected in lungs of Meriva™-treated mice (Figure 1F). In order to determine whether very low levels of curcumin or its metabolites had reached the lung following Meriva™ treatment, plasma and lung samples were incubated with β -glucuronidase/sulfatase (Figures 1G and 1H).

The retention times were: 15.8 mins for curcumin glucuronide, 19.3 mins for curcumin sulfate, and 23.7 mins for parent curcumin. The lower limit of quantification (LLOQ) of the assay was determined by assessing the lowest concentration in the standard curve that could be quantified with 80-120% accuracy and precision (coefficient of variance <20%). The LLOQ values for curcumin, glucuronide and sulfate metabolites were 0.05 μ g/mL in plasma and 0.1 μ g/mL in lung tissue. The values for accuracy and precision at the LLOQ are shown in Table 1. HPLC-UV methods with better sensitivity compared to this method as well as shorter run times have previously been published (Pak et al., 2003; Schiborr et al., 2010), however these methods detected parent curcumin only. Previous preclinical and clinical studies suggest that following curcumin administration its metabolites are the major species in plasma, with concentrations of curcumin being negligible. The method described here, separated and quantified the three species with good resolution and an adequate LLOQ, indicating its suitability for use in future pharmacokinetic studies. The ability to undertake concurrent and accurate analysis of curcumin and its major metabolites in a single run is essential for studies in which sample volumes are limited.

3.2 Accuracy and precision

For determination of accuracy and precision, plasma and lung samples spiked with curcumin, glucuronide, and sulfate metabolites (5 replicates each) at low, medium and high concentrations, were utilised. Table 2 summarises the intra- and inter-day accuracy and precision for both matrices. The criteria of $\leq 15\%$ for adequate accuracy and $\leq 15\%$ of CV for adequate precision were achieved at all concentrations examined, except for curcumin sulfate in lung at a concentration of 2 $\mu\text{g/mL}$ where the value for precision was 15.6%.

3.3 Recovery

In order to assess the recovery of curcumin and its metabolites in plasma and lung, the peak areas of analytes observed after extraction of spiked samples were compared with those seen after direct injection of known amounts of standard solutions. Three different concentrations were assessed on 3 separate occasions, and the proportion of each curcumin species recovered, was calculated. Recovery from plasma for all three analytes was in the range of 89%-105%. However, whilst recovery of curcumin from lung tissue was 98%-106%, this dropped to 53%-60% for curcumin glucuronide, and 56%-67% for curcumin sulfate (Table 2).

3.4 Linearity

Linearity was calculated using a calibration curve for each analyte (data not shown) in plasma and lung matrices. Calibration curves spanned seven concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, 5 $\mu\text{g/mL}$), and were linear for all analytes in both the matrices. The average correlation coefficient (r^2) exceeded 0.998 ($n = 3$), with accuracy values for the entire range of calibration standards of $\pm 15\%$, and thus within acceptable limits. The concentration:response relationship was continuous and reproducible.

3.5 Stability

To assess freeze-thaw stability, three different concentrations of curcumin/metabolites were used over 3 freeze-thaw cycles. Concentrations were as follows: 0.1 $\mu\text{g/mL}$ (low), 2 $\mu\text{g/mL}$ (medium) and 5 $\mu\text{g/mL}$ (high) for plasma and 0.2 $\mu\text{g/mL}$ (low), 2 $\mu\text{g/mL}$ (medium) and 5 $\mu\text{g/mL}$ (high) for lung. Both matrices were spiked with the relevant curcuminoids and stored at -80°C for 24 hours prior to thawing at room temperature. Following this, samples were then refrozen at -80°C for 24 hours, and the procedure repeated twice more. In plasma

samples, levels of curcumin and its metabolites were found to be stable after three freeze-thaw cycles, with no significant difference observed in their measured values (p values were 0.58, 0.15, and 0.38 for low, medium, and high concentration respectively). However, in lung, curcumin glucuronide degraded by up to 18%, with increased degradation observed at cycle 3 compared with cycle 1. For curcumin sulfate, the majority of degradation (up to 47%) occurred following the 1st freeze-thaw cycle. The levels of curcumin increased by 50% suggesting that curcumin metabolites have converted back to parent curcumin after multiple freeze-thaw cycles (Table 3). Long-term stability was determined by analysing the samples stored at -80°C after 3, 7, and 14 days, and 3 months. In plasma, parent curcumin decreased by up to 11%, curcumin glucuronide by up to 15%, and curcumin sulfate by up to 23%. In lung, curcumin levels decreased by up to 12%, curcumin glucuronide by up to 17%, and curcumin sulfate by up to 50% (Supplementary Table 1). The decrease observed for curcumin sulfate occurred by day 3, but did not further decrease thereafter, suggesting that the change may be a function of the freeze-thaw process rather than a temporal relationship. Whilst curcumin levels in lung appeared relatively stable, sulfate degradation may have contributed to these levels.

3.6 *In vivo* pharmacokinetic study

A study comparing the pharmacokinetics of formulated curcumin (Meriva™) and standard curcumin was undertaken to compare bioavailability at low doses in the plasma and lung. After Meriva™ or standard curcumin was given at 70 mg/kg by oral gavage, no free curcumin was detected in the plasma of any mice in either of the treatment groups. However, conjugate metabolites were detectable, with maximum concentration values ($C_{\max}$) for curcumin glucuronide and DMC glucuronide being 6- and 7-fold higher respectively, and curcumin sulfate approximately 2-fold higher, in the Meriva™ group compared to the standard curcumin group. The concentrations of the DMC glucuronide were estimated using the curcumin glucuronide calibration line since a DMC glucuronide standard was not available to construct a calibration line. In mice administered standard curcumin the time to reach peak levels ($T_{\max}$) was 0.5 h for curcumin glucuronide and DMC glucuronide, and 1 h for curcumin sulfate (Figures 2A, 2B, 2D and Table 4). In the Meriva™ group, $T_{\max}$ values were generally consistent with this, except for curcumin sulfate, which peaked 2 hours later in these animals. For both treatment groups, an unidentified peak was observed with a retention time of 12.3 minutes (peak 7 in Figure 1E). To determine the identity of the peak, samples were analysed by LC-MS/MS selected reaction monitoring (SRM); the peak was monitored for the molecular ion ($[M-H]^-$) to curcumin associated product ion fragment transition of 623>134 m/z , which was consistent with curcumin glucuronide-sulfate. (Supplementary

Figure S3). The elution time observed was also consistent with glucuronide-sulfate as a di-substituted conjugate, as these structures are more polar and would be expected to elute before the mono-substituted glucuronide and sulfate metabolites. Furthermore, this peak disappeared upon incubation with glucuronidase/sulfatase enzymes. Due to the unavailability of the appropriate standard, the curcumin calibration line was used to estimate the concentration of curcumin glucuronide-sulfate disubstituted conjugate. $C_{\max}$ for this glucuronide-sulfate conjugate was 3.5-fold higher in the Meriva™ group with a maximum concentration of ~3.15 μM reached at 1.5 hours (Figures 1E, 2C and Table 4). In lung tissue, neither curcumin nor its metabolites could be detected (Figure 1F). To determine overall curcuminoid concentrations and to ascertain whether curcumin actually reached the lungs in either its free or conjugated form, samples were subjected to enzymatic conversion. Following conversion, the Meriva™-treated mice had higher levels of both DMC (2-fold higher), and curcumin (3.8-fold higher) in plasma when compared to those animals administered standard curcumin, with average maximum concentrations of ~6.5 μM reached at 1.5 h post-dose (Figures 1G, 2E, 2F, 2G and Table 4). bDMC could not be detected in the standard curcumin group. Following enzymatic conversion, free curcumin levels in the lungs of Meriva™-treated mice were 4.6-times higher than those receiving standard curcumin, with average maximum concentrations of 0.41 μM reached 3 h after dosing (Figure 1H, 2H, 2I and Table 4). DMC was detected only in the lungs of animals that received Meriva™, whereas bDMC was undetectable in both groups. The concentrations of DMC and bDMC were estimated using the curcumin calibration line and confirmation of their identity was obtained by using LC-MS/MS SRM.

4.0 Conclusion

Here, we have developed a sensitive HPLC-UV method for determination of curcumin and its metabolites in plasma and lung tissue of mice. Hitherto, previously published pharmacokinetic studies of curcumin did not take into account the recovery of curcumin metabolites and estimated their levels using calibration curves derived from curcumin. For the first time, we report a partially validated HPLC-UV method using sulfate and glucuronide standards allowing precise quantification of curcumin metabolites, in addition to providing essential information on their stability in complex biomatrices. The method described utilises low plasma volumes of 100 μL which is of particular importance in preclinical pharmacokinetic studies where sample volume is very limited. All of the criteria, as described by FDA 2001 guidelines for the validation of bioanalytical methods, were met. The method offers a simple extraction procedure with reproducible results and. was successfully applied to an *in vivo* pharmacokinetic study. This study reveals that Meriva™ offers higher levels of curcumin

metabolites in plasma and higher levels of curcuminoids in both plasma and lung, post enzymatic conversion. In addition, curcumin glucuronide-sulfate was found to be a major metabolite of curcumin following oral administration, which has not been previously reported. The authors recognise that use of a curcumin calibration line to estimate levels of the curcumin glucuronide-sulfate conjugate is a limitation of this current method, as with this approach, the actual concentration of the analyte may vary slightly. However, a standard for this analyte was not readily available, nor had there been previously published methodology to facilitate its synthesis in-house. To our knowledge presence of this analyte has not been reported in previous *in vivo* studies. The data presented sheds light on the relative instability of glucuronide and sulfate metabolites in the lung matrix following multiple freeze-thaw cycles. One of the approaches by which this could be addressed in future studies, would be to identify an appropriate analyte stabiliser which would enhance provision of more robust validation data sets. In conclusion, this method is suitable for the simultaneous determination of curcumin and its metabolites in low dose preclinical studies.

5.0 Acknowledgments

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Conflict of interest

Part of this work was supported financially by Indena S.P.A, Milan. Indena did not have input into methodological design, data analysis, or this published article.

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Figure legends:

Figure 1. Representative HPLC chromatograms of mouse plasma and lung following oral gavage dosing of Meriva™.

HPLC-UV chromatograms of plasma and lungs taken from mice receiving Meriva™ at 70 mg/kg in terms of curcumin dose by oral gavage obtained 1 hour post dosing. The chromatograms are as follows: (A) Plasma extract from vehicle control mouse (B) Lung Extract from vehicle control mouse (C) Extract from plasma spiked with curcumin, curcumin sulfate and curcumin glucuronide standards at 2 µg/mL (D) Extract from lung spiked with curcumin, curcumin sulfate and curcumin glucuronide standards at 2 µg/mL. (E) Plasma extract from mouse receiving Meriva™. (F) Lung extract from mouse receiving Meriva™. (G) Enzymatically converted plasma extract from mouse receiving Meriva™. (H) Enzymatically converted lung extract from mouse receiving Meriva™. Peaks 1-7 correspond to: (1) curcumin, (2) demethoxycurcumin, (3) *bis*-demethoxycurcumin, (4) curcumin sulfate (5) curcumin glucuronide, (6) demethoxycurcumin glucuronide, (7) curcumin glucuronide-sulfate. A similar eluting chromatogram was observed for standard curcumin (Supplementary Fig. 2). Absorbance was monitored at 426 nm.

Figure 2. Pre and post-enzymatic conversion concentration value of curcuminoids and curcuminoid metabolites in mouse plasma and lung following oral dosing with unformulated curcumin or Meriva™.

Pre-enzymatic conversion plasma levels of (A) curcumin glucuronide, (B) demethoxycurcumin glucuronide, (C) curcumin glucuronide-sulfate and (D) curcumin sulfate. Post enzymatic conversion plasma levels of (E) curcumin, (F) demethoxycurcumin and (G) *bis*-demethoxycurcumin. Post enzymatic conversion lung levels of (H) curcumin and (I) demethoxycurcumin. Values are the mean + SD (n = 3). Statistical comparison between the Meriva™ and standard curcumin at each time point was by student's T-test. Statistically significant differences are indicated as * (p< 0.05) and *** (p< 0.005).

Supplementary Figure 1. Structure of curcuminoids.

Supplementary Figure 2. Representative HPLC chromatograms of mouse plasma and lung following oral gavage dosing of standard curcumin.

HPLC-UV chromatograms of plasma and lungs taken from mice receiving unformulated curcumin at 70 mg/kg by oral gavage obtained 1 hour post dosing. The chromatograms are as follows: (A) Plasma extract from mouse receiving standard curcumin. (B) Enzymatically converted plasma extract from mouse receiving standard curcumin. (C) Lung extract from mouse receiving standard curcumin. (D) Enzymatically converted lung extract from mouse receiving standard curcumin. Peak 1-6 correspond to: (1) curcumin, (2) demethoxycurcumin, (3) curcumin sulfate (4) curcumin glucuronide, (5) demethoxycurcumin glucuronide, (6) curcumin glucuronide-sulfate. Absorbance was monitored at 426 nm.

453 **Supplementary Figure 3. LC-MS/MS SRM and UV analysis of the HPLC-UV peak eluting at 12.3 min**
454 **following treatment of mice with standard curcumin or Meriva™.** The molecular ion ($[M-H]^-$) to curcumin
455 associated product ion fragment transition of $623>134m/z$ was monitored, which was consistent with the
456 presence of curcumin glucuronide-sulfate.

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Table 1 Assay accuracy and precision for the measurement of curcumin, curcumin glucuronide and curcumin sulfate at the lower limit of quantification (LLOQ)

	LLOQ (µg/mL)	Plasma (<i>n</i> =5)		LLOQ (µg/mL)	Lung (<i>n</i> =5)	
		Accuracy (%) ^a	Precision (% CV)		Accuracy (%) ^a	Precision (% CV)
Curcumin	0.05	101.4±4.7	4.5	0.1	101.1±12.1	12.0
Curcumin Glucuronide	0.05	94.4±8.7	9.2	0.1	112.8±14.6	12.9
Curcumin Sulfate	0.05	97.3±6.0	6.2	0.1	103.6±13.6	13.2

^a Values are mean ± SD of *n*=5 determinations

Table 2 Recovery, intra-day and inter-day accuracy and precision for determination of curcumin, curcumin glucuronide and curcumin sulfate in mouse plasma and lung homogenate.

Analyte	Concentration ($\mu\text{g/mL}$)	Recovery (%) ^a	Inter-day		Intra-day	
			Accuracy (%) ^a	Precision (% CV)	Accuracy (%) ^a	Precision (% CV)
Plasma						
Curcumin	0.1	93.3 $\pm$ 6.7	90.1 $\pm$ 13.5	14.8	86.3 $\pm$ 10.4	11.6
	2	94.8 $\pm$ 6.9	84.6 $\pm$ 9.2	10.9	89.3 $\pm$ 9.3	10.1
	5	88.7 $\pm$ 4.6	90.5 $\pm$ 9.9	10.9	106.1 $\pm$ 3.83	3.6
Curcumin Glucuronide	0.1	90.7 $\pm$ 11.0	94.4 $\pm$ 8.7	9.2	105.0 $\pm$ 8.6	8.2
	2	90.2 $\pm$ 4.1	89.7 $\pm$ 3.6	4.1	96.6 $\pm$ 4.8	5.0
	5	92.2 $\pm$ 5.1	100.5 $\pm$ 8.7	8.6	108.8 $\pm$ 8.0	7.4
Curcumin Sulfate	0.1	104.5 $\pm$ 13.2	97.5 $\pm$ 6.0	6.2	92.1 $\pm$ 6.9	10.3
	2	94.2 $\pm$ 9.3	86.5 $\pm$ 4.1	4.7	88.4 $\pm$ 5.5	6.2
	5	95.0 $\pm$ 7.5	95.7 $\pm$ 3.6	3.8	90.4 $\pm$ 4.4	4.8
Lung						
Curcumin	0.5	105.8 $\pm$ 2.75	107.2 $\pm$ 12.8	12.0	91.9 $\pm$ 4.3	9.1
	2	98.2 $\pm$ 3.4	98.5 $\pm$ 5.6	5.7	93.2 $\pm$ 7.9	7.3
	5	97.6 $\pm$ 6.5	93.8 $\pm$ 10.3	11.6	98.5 $\pm$ 5.7	11.6
Curcumin Glucuronide	0.5	54.2 $\pm$ 2.2	112.0 $\pm$ 11.1	9.9	113.3 $\pm$ 3.9	12.9
	2	53.3 $\pm$ 5.1	87.6 $\pm$ 10.8	12.3	97.4 $\pm$ 9.6	3.9
	5	60.1 $\pm$ 3.8	102.8 $\pm$ 4.5	4.4	95.7 $\pm$ 15.0	3.1
Curcumin Sulfate	0.5	56.4 $\pm$ 2.3	105.7 $\pm$ 9.1	8.6	112.8 $\pm$ 2.4	8.6
	2	62.2 $\pm$ 2.0	101.4 $\pm$ 15.9	15.6	99.3 $\pm$ 3.6	12.3
	5	66.9 $\pm$ 7.1	97.7 $\pm$ 10.0	10.2	95.34 $\pm$ 4.8	10.2

^a Values are mean $\pm$ SD of $n=5$ determinations.

Table 3. Freeze-thaw stability of curcumin, curcumin glucuronide and curcumin sulfate in mouse plasma and lung homogenate. Samples were frozen at -80°C and thawed unassisted at room temperature once for cycle 1 and thrice for cycle 3.

Plasma	Analyte	Concentration (µg/mL)	Content measured (%) ^a	
			Cycle 1	Cycle 3
	Curcumin	0.1	96.0±0.9	97.4±4.8
		2	96.5±7.1	100.0±1.5
		5	108.6±14.3	102.3±1.6
	Curcumin Glucuronide	0.1	107.0±12.1	101.3±9.3
		2	103.5±10.7	96.5±1.3
		5	97.2±3.5	96.0±3.4
	Curcumin Sulfate	0.1	101.2±4.0	118.3±3.9
		2	98.3±13.6	91.2±1.7
		5	101.3±10.6	108.2±5.0
Lung	Curcumin	0.5	98.2±5.4	149.9±12.5
		2	102.2±6.9	126.5±16.8
		5	100.3±3.4	149.3±4.2
	Curcumin Glucuronide	0.5	97.2±4.6	81.8±7.0
		2	99.5±10.0	91.2±0.3
		5	92.0±7.6	82.2±5.1
	Curcumin Sulfate	0.5	62.7±6.6	53.4±5.7
		2	50.3±4.8	62.9±2.0
		5	66.5±6.2	63.6±1.4

^a Values are mean ± SD of *n*=5 determinations.

Table 4. Average curcuminoid concentrations in mouse plasma and lungs after 70 mg/kg oral dosing (pre- and post enzymatic conversion).

Estimated average plasma peak levels ($C_{\max}$), time of peak levels ($T_{\max}$) and AUC values for standard curcumin and Meriva™ when administered at a dose of 70 mg/kg in terms of curcumin, via oral gavage.

Plasma				
		$C_{\max}$ (μ M)	$T_{\max}$ (h)	AUC (nmol/h/mL)
Standard Curcumin	Curcumin glucuronide	0.41±0.29	0.5	1.7
	Demethoxycurcumin glucuronide ^b	0.39±0.30	0.5	3.25
	Curcumin sulfate	0.24±0.11	1	0.08
	Curcumin glucuronide-sulfate ^a	0.9±0.2	1.5	3.41
Meriva™	Curcumin glucuronide	2.61±1.89	0.5	9.77
	Demethoxycurcumin glucuronide ^b	2.81±0.87	0.25	12.54
	Curcumin Sulfate	0.46±0.023	3	1.12
	Curcumin glucuronide-sulfate ^a	3.15±0.45	1.5	10.28
Plasma (Post enzymatic conversion)				
		$C_{\max}$ (μ M)	$T_{\max}$ (h)	AUC (nmol/h/mL)
Standard	Curcumin	1.71±0.57	1.5	3.29
	Demethoxycurcumin ^c	1.00±0.04	1	1.41
Meriva™	Curcumin	6.53±3.48	1.5	14.14
	Demethoxycurcumin ^c	2.29±0.32	0.25	4.32
	<i>bis</i> -Demethoxycurcumin ^c	1.18±1.00	0.25	0.62
Lungs (Post enzymatic conversion)				
Standard	Curcumin	0.09±0.01	3	0.20
Meriva™	Curcumin	0.41±0.05	3	1.07
	Demethoxycurcumin ^c	0.21±0.06	0.25	0.41

No free curcumin or its metabolites were detected in lung tissue when samples were directly analysed without enzymatic conversion. Values are mean ± SD, with 3 animals per time point. ^aCurcumin glucuronide-sulfate concentrations were estimated using the curcumin calibration line. ^bDemethoxycurcumin glucuronide concentrations were estimated using the curcumin glucuronide calibration line. ^cDemethoxycurcumin and *bis*-Demethoxycurcumin concentrations were estimated using the curcumin calibration line.