Bioavailability of Curcumin and Curcumin Glucuronide in the Central Nervous System of Mice after Oral Delivery of Nano-Curcumin

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Abstract

Curcumin is a bioactive molecule extracted from Turmeric roots that has been recognized to possess a wide variety of important biological activities. Despite its great pharmacological activities, curcumin is highly hydrophobic, which results in poor bioavailability. We have formulated this hydrophobic compound into stable polymeric nanoparticles (nano-curcumin) to enhance its oral absorption. Pharmacokinetic analysis after oral delivery of nano-curcumin in mice demonstrated approximately 20-fold reduction in dose requirement when compared to unformulated curcumin to achieve comparable plasma and central nervous system (CNS) tissue concentrations. This investigation corroborated our previous study of curcumin functionality of attenuating opioid tolerance and dependence, which shows equivalent efficacy of low-dose (20 mg/kg) nano-curcumin and high-dose (400 mg/kg) pure curcumin in mice. Furthermore, the highly selective and validated liquid chromatography-mass spectrometry (LC-MS) method was developed to quantify curcumin glucuronide, the major metabolite of curcumin. The results suggest that the presence of curcumin in the CNS is essential for prevention and reversal of opioid tolerance and dependence.

Keywords: nano-curcumin, oral delivery, bioavailability, central nervous system (CNS), liquid chromatography-mass spectrometry (LC-MS)

1. Introduction

Curcumin is a naturally occurring phenolic compound that can be isolated from Turmeric roots. In recent years, a plethora of interesting and very promising bioactivities has been identified or attributed to curcumin, notably antioxidant,^{1, 2} anti-inflammatory,³ antimicrobial,^{4, 5} and neuroprotective⁶ properties. Our previous studies also suggested that curcumin could attenuate or even reverse opioid antinociceptive tolerance and dependence in mice.^{7, 8} However, the hydrophobic nature of the drug limits its aqueous solubility and bioavailability, hindering curcumin's clinical translation into a useful therapeutic agent.

Several strategies have been employed to increase solubility and oral bioavailability of curcumin. Chemical conjugation of curcumin with phospholipids, proteins, or polysaccharides has been demonstrated as one way to enhance its solubility.^{9, 10} The use of adjuvant molecules such as piperine, quercetin, or silibinin and their concomitant administration with curcumin has been implemented and shown to improve the oral absorption of curcumin by inhibiting metabolic conjugation such as conjugation with glucuronic acid.^{11, 12} However, in most of these cases curcumin is not fully protected and could be rapidly degraded. Furthermore, the strategies discussed above do not provide the ability to target curcumin into specific sites of action. More recently, encapsulation of curcumin in polymeric nanoparticles and lipid nanocarriers has been extensively studied to develop formulations that could not only improve solubility of the drug, but also provide protection from enzymatic and pH degradation, prolong its circulation inside the body after initial oral absorption, and with additional surface modification (i.e. attachment of targeting molecules such as monoclonal antibodies and aptamers) provide sustained drug release at the target tissue. Nanoparticles increase the dissolution rate of the hydrophobic compounds by providing large surface-to-volume ratio. Moreover, the solubility of the compounds depends on

the size of the particles when they are smaller than 100 nm due to the curvature effect.^{13, 14} The most common polymeric carriers for curcumin reported in literature include poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-*b*-PLA), and poly(ethylene glycol)-*b*-poly(lactic-co-glycolic acid) (PEG-*b*-PLGA) polymeric particles.¹⁵⁻¹⁸ Curcumin encapsulated in phospholipid vesicles composed of soybean lecithins (SLP-WHITE and SLP-PC70, 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dimyristoyl-sn-glycero-3-phospho-*rac*-(1-glycerol) (DMPG)) has also been investigated.¹⁹⁻²¹ However, the methods that are used for the production of the aforementioned formulations (such as emulsion-based encapsulation or co-solvent evaporation) suffer from low drug loading (<15%) and broad particle size distribution.

We developed a flash-nanoprecipitation-and-drying process to manipulate non-equilibrium nanostructures via kinetic control of the flow field and polymer micellization. The nanoparticles in dry-powder format could be stable more than 12 months. Rapid co-precipitation of hydrophobic compounds and block copolymers overcomes thermodynamic limit of drug loading, resulting in “kinetically frozen” nanostructures with high drug loading (~50%) and almost 100% encapsulation efficiency. Furthermore, our process is highly reproducible and scalable for the increased demand of curcumin nanoparticles.^{22, 23}

Employing the nano-curcumin (curcumin encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles) as the platform, we have previously demonstrated the efficacy of nano-curcumin in both prevention and reversal of morphine tolerance and dependence in mice at a relatively low oral dose.^{7, 8} Furthermore, it was determined that the observed behavioral effects in the rodent models correlated with curcumin's inhibition of CaMKII α activity in the brain, suggesting an underlying mechanism involving the presence of curcumin in the central nervous

system (CNS) to suppress the activity of CaMKII α that is critically important in the development and maintenance of opioid tolerance and dependence.^{7, 24, 25} A pharmacokinetic study was then necessary to quantify curcumin in the CNS and in blood plasma after its oral administration, so that nanoparticle format of curcumin could be further adopted as the platform to completely elucidate the underlying mechanism and to eventually become an essential part of a tool kit for severe pain management.

Although many studies reported the pharmacokinetics of curcumin in rodents or humans, a lot of them do not separately analyze curcumin and its metabolites, causing overestimation of curcumin availability in blood or tissues. It has been observed that these studies usually lack the analytical details such as HPLC/LCMS chromatogram data or blood/tissue collection and treatment protocols. Since curcumin can be quickly metabolized, the lack of specificity may mislead investigators when designing nanoparticle formulations and evaluating the therapeutic mechanisms and efficacy.

In this work, curcumin bioavailability in plasma, brain and for the first time spinal cord tissue after oral administration of nano-curcumin and curcumin was investigated by employing a highly sensitive LCMS/MS method. Furthermore, the examination of the main curcumin metabolite, curcumin glucuronide, upon oral administration, was conducted using a novel methodology of direct extraction and quantification without hydrolyzing the glucuronide moiety. The obtained results correlate well with our previous reports of nano-curcumin's efficacy of attenuating morphine tolerance and dependence based on behavioral studies in mice models.

2. Materials and Methods

2.1. Materials and Reagents

PLGA (acid terminated; PLA:PGA 50:50 w/w; Mw 7000-17000, Mw/Mn=1.8), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), trehalose, leucine, methanol, acetonitrile, and ethyl acetate were purchased from Sigma-Aldrich (St Louis, MO). Curcumin (>98%) was purchased from ChromaDex (Irvine, CA). Curcumin glucuronide standard was purchased from TLC PHARMACHEM (Ontario, Canada).

2.2. Animals

Male ICR mice (20-25 g, Harlan Laboratories, Indianapolis, IN) were maintained on a 14/10h light/dark cycle with access to food and water *ad libitum* prior to experimental procedures. All experiments were performed with an approval by the Animal Care and Use Committee of the University of Illinois at Chicago and in accordance with the policies and recommendations of the National Institutes of Health guidelines for the handling and use of laboratory animals.

2.3. Nanoparticle preparation and characterization

Nanoparticles of curcumin encapsulated in PLGA were generated by flash nanoprecipitation using a multi-inlet vortex mixer (MIVM), as previously described.⁸ One of the four inlet streams was 2 wt% PLGA together with 2 wt% curcumin dissolved in THF. The other three inlet streams consisted of deionized water as an anti-solvent to precipitate both the drug compound and the polymer. The flow rate of stream 1 (organic stream) and 2 was set to be 6 ml/min and 54 ml/min for streams 3 and 4. Nanoparticles (40 ml) were collected into a beaker containing 3.5 ml solution of 428.6 mg/ml trehalose and 1.5 ml solution of 15 mg/ml leucine in deionized water under constant stirring. The collected nanoparticle suspensions were flash frozen in an ethanol-dry ice bath and further freeze-dried to dry-powder format.

To measure drug loading (DL) and encapsulation efficiency (EE) of curcumin in

nanoparticles, the nanoparticle suspensions were filtered with 0.45 μm filters before being freeze-dried. The unloaded curcumin quickly forms micron-sized or larger particles that would be removed completely during the filtration step. The curcumin in the stable particle format was considered as encapsulated. The dried powder was re-dissolved in DMSO at the solid construct concentration of 10 mg/ml. The amount of curcumin was quantified by using UV-Vis spectrophotometry at the absorbance wavelength of 420 nm.

$$\text{DL (\%)} = \frac{\text{Amount of curcumin encapsulated in nanoparticles}}{\text{Total weight of nanoparticles}} \times 100\%$$

$$\text{EE (\%)} = \frac{\text{Amount of curcumin encapsulated in nanoparticles}}{\text{Feeding amount of curcumin}} \times 100\%$$

Nanoparticle size distribution right after nanoprecipitation and after resuspension of dried powder in deionized water were measured by dynamic light scattering (DLS) (Brookhaven, NanoDLS, Holtsville, NY). The powder was resuspended to reach final curcumin concentration of 1.0 mg/ml, which was about 10 times more concentrated than the original nanosuspension before freeze-drying. The size of the particles was reported as the intensity-weighted diameter. The values for viscosity and refractive index were set to be 0.890 cP and 1.331, respectively.

2.4. Freeze drying of the nanoparticle suspensions and resuspension of the powders into nanoparticles

To maintain long-term stability of the nanoparticles and to conveniently resuspend nanoparticles at various curcumin concentrations, freeze drying has been applied and optimized. Moreover, it is ensured that the majority of organic solvent was removed after the drying process. Trehalose and leucine were added to the nanoparticle suspensions, serving as spacers in

order to prevent the nanoparticles from permanent aggregation. The nanoparticles could be resuspended homogeneously at the ratios of nanoparticle to trehalose and leucine equivalent to or greater than 1:200 and 1:3, respectively.

The freeze-drying process lasted 2-3 days in a freeze dryer (Labconco, FreeZone 1 Liter Console Freeze Dry Systems, Kansas City, MO) at a vacuum pressure and -47 °C. The dried powders were resuspended at high construct concentrations by using a water bath sonicator for 5 minutes (Branson, CPX1800, Taiwan) and immediately dosed to mice.

2.5. Pharmacokinetic study in mice

2.5.1. Dosing and plasma and tissue collection

Separate groups of mice received nano-curcumin (PLGA nanoparticles encapsulating curcumin) by gastric gavage at a dose of 20 mg/kg or aqueous suspension of pure curcumin at a dose of 20 mg/kg and 400 mg/kg. At eight different time points (3 min, 5 min 7.5 min, 15 min, 30 min, 1 hr, 2 hr, and 4 hr), four mice per time point were sacrificed and the blood was withdrawn via cardiac puncture with a heparinized syringe. The collected blood samples were centrifuged at 3,000 rpm for 10 minutes to separate plasma. Immediately following blood collection, perfusion with normal saline was performed. Brain and spinal cord tissues were collected, rinsed with ice-cold saline and stored at -80 °C until further analysis. At the day of analysis all tissues were homogenized with normal saline.

2.5.2. Extraction of curcumin and curcumin glucuronide from plasma and homogenized tissues

The extraction of curcumin from plasma, brain and spinal cord was performed by liquid-liquid extraction with ethyl acetate. Briefly, 1000 µl of ethyl acetate was added into 125 µl of

plasma or tissue homogenate and vortexed at high speed for 5 minutes. The mixture was then centrifuged at 3500 rpm for 10 minutes to achieve a suitable phase separation. The supernatant was collected. A different extraction protocol was used for the metabolite since ethyl acetate was not an efficient solvent for its extraction. Briefly, 62.5 μ l of 5M ammonium acetate was added into 125 μ l plasma or tissue homogenate and mixed for 1 min. 1000 μ l of acetonitrile was added into each sample and vortexed for 5 min. The mixture was then centrifuged at 3500 rpm for 10 minutes and the supernatant was collected. The supernatant was evaporated by a vacufuge (Eppendorf, Hauppauge, NY) and reconstituted with a smaller amount of methanol. The concentrated samples were analyzed using a liquid chromatography – mass spectrometry (LC-MS) and a high-performance liquid chromatography (HPLC) as described in the next section. Standards were prepared by spiking a known concentration of curcumin, curcumin glucuronide, and hesperetin (IS) into plasma or tissue homogenates followed by the same extraction protocol described above. The extraction efficiencies were calculated and reported in the **Supporting Information** section (Table S1 & S2).

2.5.3. HPLC and LCMS analysis

HPLC analysis was carried out on a Shimadzu HPLC system (Columbia, MD). The chromatographic separation was performed on a Thermo Scientific Hypersil Gold C18 column (2.1 x 50 mm, 5 μ m). Liquid chromatographic (LC) conditions consisted of a gradient method starting with 5% acetonitrile/95% 0.1% formic acid and reaching 95% acetonitrile/5% 0.1% formic acid in water over 8 minutes, followed by 4 minutes of equilibration. The flow rate was set to be 0.3 ml/min. The wavelength of detection was at 420 nm.

The scanning instrument Shimadzu LCMS (LC20ADXR)-IT-TOF (Columbia, MD) that couples atmospheric pressure ionization with Ion –Trap (IT) and Time-of-Flight (TOF)

technologies was used to identify curcumin and curcumin metabolite. Liquid chromatographic (LC) conditions consisted of a gradient method starting with 5% acetonitrile/95% 0.1% formic acid in water and reaching 100% acetonitrile over 10.5 minutes, followed by 4.5 minutes of equilibration. Thermo Scientific Hypersil Gold C18 column (2.1 x 50 mm, 5 μ m) was used for the measurements. The flow rate was set to be 0.25 ml/min. Photodiode array (PDA) was scanned 200-800 nm over the entire LC run. MS was scanned (m/z 100-1000) in both positive and negative mode.

Both curcumin and curcumin glucuronide were quantified by negative ion tandem mass spectroscopy. Analysis was performed by using Triple Quad LC Mass Spectrometer (Agilent, 6410 QQQ, Santa Clara, CA). Separation was achieved with the same conditions as described above for LCMS-IT-TOF. Identification and quantification of curcuminoids was done by multiple reaction monitoring (MRM) using suitable transitions (curcumin 367->216.9, curcumin glucuronide 543->175 and hesperetin (IS) 301.1>163.9) after initial full scan acquisition. Other obtained ion transitions for curcumin (367.1->148.9) and curcumin glucuronide (543->217 and 543->367) were used as qualifiers.

3. Results and Discussion

We have previously developed a continuous, scalable process by integrating flash precipitation and drying processes to generate stable nanoparticles with high drug loading and narrow size distribution.^{22, 23} Nano-curcumin (curcumin-PLGA nanoparticles) was produced adopting this method. The size of the nanoparticle was characterized by DLS and reported in Figure 1. The drying process caused certain level of permanent aggregation, so the particle size distribution is broader and the average size shifted from ~100 nm to ~125 nm after resuspension

(Figure 1). However, the particles are still in the range from 50 nm to 250 nm. After the freeze-drying process, the particles are in powder format, which can be maintained stable for more than a year. The calculated drug loading and encapsulation efficiency were 47.3% and 94.7%, respectively. The final amount of curcumin in the dried-powder (including nanoparticles and excipients) was 2.4 µg/mg.

Pharmacokinetic studies after oral delivery of curcumin or curcumin formulations have been commonly performed to determine bioavailability of the compound in blood and tissues. However, there has been some controversy regarding the reported results. First, curcumin is known to be highly metabolized through both intestinal and hepatic glucuronidation,^{26, 27} which implies a significant biotransformation after oral administration. Literatures have reported the improved bioavailability of curcumin in plasma that was pre-treated with β-glucuronidase, resulting in hydrolysis of the glucuronide moiety.^{19, 28, 29} Detected levels of curcumin if including its metabolites do not really represent the true bioavailability of the native drug and could be misleading since the activity of the metabolites could be significantly altered as suggested by several *in vitro* studies.^{30, 31} Furthermore, the lack of detailed protocols in various pharmacokinetic studies regarding the sample pre-treatment and HPLC or LCMS procedures that actually reported larger quantities of curcumin in plasma makes it extremely hard to understand whether curcumin was measured individually or as a combination of native drug and its metabolites.^{18, 32}

The majority of the published work on pharmacokinetic studies of native curcumin in both rodent and human models has consistently shown poor oral bioavailability of the compound as indicated by very low or even undetectable concentrations in plasma. When aqueous suspension of curcumin was given at a relatively high dose of 1 g/kg in rats, the maximum

detectable plasma concentration was 22 ng/ml.¹⁷ On the other hand, a much lower dose (50mg/kg) of curcumin-loaded PLGA nanoparticles has shown curcumin maximum rat plasma level of 44 ng/ml indicating improved bioavailability.¹⁷ A separate study of curcumin level in rat plasma after oral administration of curcumin loaded in low molecular weight - PLGA (MW: 5-15k) nanoparticles at 50 mg/kg curcumin dose reported a peak plasma concentration of 42 ng/ml at 30min after administration, which rapidly vanished to concentrations below 20 ng/ml after 1hr.¹⁵ To the best of our knowledge only one article besides our study reported a very low dose of curcumin (23 mg/kg) encapsulated in PEG-PLA nanoparticles used to measure curcumin bioavailability in blood plasma after oral administration in mice.³³ In this study, dried PEG-PLA – curcumin nanoparticles were administered to mice to observe the time dependent curcumin and tetrahydrocurcumin levels in plasma and brain tissue. It was established that at an early time point of 10 min, the mean concentration of curcumin in plasma was found to be quite high (400 ng/ml) but with large standard deviation. However, it then quickly diminished to approximately 10 ng/ml levels around 40 min. The curcumin level in brain for this low oral dose peaked at 20 ng/g, which is an evidence that curcumin crossed the blood-brain barrier (BBB).³³

The dose tested here for our nano-curcumin is relatively low (20 mg/kg) as this is a feasible amount (per kg of the body weight) of curcumin that could be safely administered daily in humans without undesired side effects.³⁴ Furthermore, we have recently demonstrated that nano-curcumin at this relatively low dose (20 mg/kg) was highly efficacious in attenuating morphine tolerance and dependence in rodent models.^{7, 8} At the same dosing level, negligible amount of curcumin was detected after oral administration of the unformulated curcumin. As a comparison, a much higher dose (400 mg/kg) of aqueous suspension of native curcumin has to be used to achieve comparable amount of curcumin in plasma, brain, and spinal cord.

The quantification of curcumin and curcumin glucuronide was obtained and extrapolated on the calibration curve. The calibration lines were shown to be linear from 0.122 ng/ml to 250 ng/ml ($r^2 > 0.99$) for curcumin and 0.98 ng/ml to 2000 ng/ml ($r^2 > 0.991$) for curcumin glucuronide standards extracted from plasma, brain and spinal cord homogenates (Figure S3). Internal standard calibration was also used to quantify the results (Figure S3B and S3D). However, no significant difference in data quality was found when comparing both internal and external standardization (Figure S4). The specificity of the method was further confirmed by comparing chromatograms of the control samples (curcumin extracted from the mixture of blank plasma/brain/spinal cord with curcumin standards) and the measured samples (curcumin extracted from plasma/brain/spinal cord after oral delivery of nano-curcumin). The example of the specificity, demonstrating that there is no interference from the brain and spinal cord matrix components, is presented in Figure 2. The figure also clearly demonstrates that curcumin infiltrated through the blood-brain barrier (BBB) as it is present in the brain and the spinal cord at early time-points when the mice were administered with a small dose (20 mg/kg) of nano-curcumin.

The amount of curcumin measured in the CNS at various time points is presented in Figure 3A and B. The LCMS analysis reveals comparable curcumin levels in both brain and spinal cord tissues after oral delivery of low dose (20 mg/kg) nano-curcumin and high dose (400 mg/kg) native curcumin. This correlates with our previous mouse behavioral studies that showed similar efficacy of low dose nano-curcumin (20 mg/kg) and high dose curcumin (400mg/kg) on blocking morphine tolerance and dependence.^{7, 8} In order to compare with the efficacy studies in rodent animals, it is important to unambiguously determine the amount of curcumin in CNS within 15 minutes after dosing. This early pharmacokinetic time frame is of great importance

since the effects on morphine tolerance and dependence reversal and prevention in both acute and chronic models were observed when nano-curcumin or pure curcumin were administered orally 15 min prior to tested doses of morphine.⁷ The presence of curcumin in both brain and spinal cord in the first 30 minutes after the dosages (Figure 3) provides another support of our hypothesis that curcumin in the CNS suppresses the function of CaMKII α that is implicated in the development of opioid tolerance and dependence.³⁵ The curcumin plasma levels are reported in Figure 3C. The results clearly indicate that curcumin is readily absorbed in the blood stream and rapidly distributed in the tissues due to its lipophilic nature. The fast decrease of curcumin concentration in plasma is also an indication of its quick metabolism.

The critical pharmacokinetic parameters including the peak concentration ($C_{\max}$), the time of the peak concentration ($T_{\max}$), and the area under the curve (AUC) were presented in Table 1. The area under the curve for the investigated time (0-250 min) (AUC_{0-250}) was calculated using a linear trapezoid rule. The AUC_{0-250} values for nano-curcumin (20 mg/kg) in plasma, brain, and spinal cord were approximately 78, 3, and 6 times higher when compared with the unformulated curcumin at the same dose (20 mg/kg). Khalil et al. performed pharmacokinetic analysis after oral delivery of curcumin-loaded PLGA nanoparticles in rats at a relatively low curcumin dose of 50 mg/kg. The study recorded a 15.6-fold increase in the AUC in plasma when compared to the unformulated curcumin at the same dose. The $C_{\max}$ was found to be 11.78 ng/ml which was lower than the $C_{\max}$ observed in our study (41.3 ng/ml).¹⁶ Another study looking at the curcumin concentration in rat plasma after oral administration of curcumin formulated with phosphatidylcholine observed a 5.6-fold increase in the AUC compared to the free form of the drug. The $C_{\max}$ and $T_{\max}$ were 33.4 ng/ml and 15 minutes, respectively.³⁶ The study of *in vivo* performance of curcumin-loaded mPEG-PLA/TPGS mixed micelles (75 mg/kg) in rats showed

the enhanced oral bioavailability as indicated by approximately 9-times increase in the AUC with the $C_{\max}$ of 197.9 ng/ml.³⁷ Other formulation strategies such as nanoparticles composed of PAAm-g-XG grafted copolymer loaded with curcumin and molecular complexes of curcumin with a pH sensitive cationic Eudragit® EPO polymer also reported several-fold increase in curcumin bioavailability compared with the unformulated drug.^{38, 39} Although the aforementioned formulation technologies have been capable of increasing bioavailability of curcumin, the majority of them require more complex and longer processing steps, and result in much lower drug loading.

Table 1. Pharmacokinetic parameters of curcumin levels in plasma, brain, and spinal cord after oral delivery of nano-curcumin (20 mg/kg) and pure curcumin suspensions (20 mg/kg and 400 mg/kg).

	20 mg/kg nano-curcumin	20 mg/kg pure curcumin	400 mg/kg pure curcumin
Brain			
$T_{\max}$ (min)	3.00	15.00	5.00
$C_{\max}$ (ng/g)	12.71±6.63	2.03±0.69	30.32±3.10
AUC ₀₋₂₄₀ (ng/g x min)	185.55±71.56	58.86±31.12	252.52±110.71
Spinal cord			
$T_{\max}$ (min)	7.50	7.50	5.00
$C_{\max}$ (ng/g)	85.88±54.27	23.49±11.57	129.16±63.12
AUC ₀₋₂₄₀ (ng/g x min)	2878.64±973.82	454.59±237.51	5154.62±2055.07
Plasma			
$T_{\max}$ (min)	3.00	15.00	3.00
$C_{\max}$ (ng/ml)	41.33±16.03	0.60±0.44	79.82±49.00
AUC ₀₋₂₄₀ (ng/ml x min)	686.14±366.22	8.73±5.43	618.83±269.04

The LCMS analysis also proved that curcumin was quickly metabolized after oral administration. Larger quantities of curcumin glucuronide reaching the order of $\mu\text{g/ml}$ levels were detected in plasma after oral dosage of nano-curcumin. On the other hand, low-dose and high-dose of native curcumin resulted in much lower concentrations of the metabolite with maximum concentrations of 10 ng/ml and 282 ng/ml, respectively (Figure 4B). The area under the concentration curve increased approximately 450 and 4.7 times for nano-curcumin when compared with the unformulated curcumin at the same dose and 20 times higher dose, respectively (Table 2). The most likely reason is that higher and faster absorption rates of curcumin in the nanoparticle format resulted in larger amount of curcumin present in blood at the much earlier time point, which could be quickly metabolized. No metabolite was detected in brain or spinal cord tissues. It is also important to note that the method used here for the quantification of curcumin metabolite allows for its direct measurement without the need for the hydrolysis of the glucuronide moiety. This offers a much more robust and reliable way of compound characterization with the benefits of rapid sample preparation and better accuracy, as the hydrolysis may result in incomplete deconjugation and non-specific matrix binding in the hydrolytic environment.

Table 2. Pharmacokinetic parameters for curcumin glucuronide in plasma after oral administration of low-dose (20 mg/kg) nano-curcumin, low-dose (20 mg/kg) pure curcumin, and high-dose (400 mg/kg) pure curcumin.

Plasma	20mg/kg nano-curcumin	20mg/kg pure curcumin	400mg/kg pure curcumin
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$T_{\max}$ (min)	7.50	30	60.00
$C_{\max}$ ($\mu\text{g/ml}$)	3.38 ± 1.04	0.01 ± 0.00	0.28 ± 0.05
AUC_{0-240} ($\mu\text{g/ml} \times \text{min}$)	184.24 ± 52.55	0.41 ± 0.07	39.60 ± 9.22

It is important to precisely detect the level of curcumin glucuronide separately. The *in vitro* efficacy of curcumin glucuronide has been investigated by several groups. However, no empirically satisfactory conclusion has been drawn regarding its potency. It has been observed that while curcumin exhibited a great anticancer activity resulting in 100% cell death, its metabolite (curcumin glucuronide) did not show any suppression of cell proliferation against KBM-5, U266, or Jurkat cell lines.³⁰ Shoji et al. compared the effects of curcumin and curcumin glucuronide in human hepatocellular carcinoma (HepG2) cells and found much weaker effects of the metabolite when compared with the native drug. The difference in the activity between curcumin and curcumin glucuronide was mainly caused by the different rate of cell uptake of the compounds.⁴⁰ On the contrary, some initial investigations suggest that polyphenol glucuronide compounds could play an active role in the treatment of some of the diseases and medical conditions through natural deconjugation into a parent compound at the site of inflammation or necrotic regimes of certain types of human tumors.⁴¹⁻⁴³ The mechanism allowing for this action involves overexpression of β -glucuronidase – an enzyme that is responsible for cleavage of the glucuronide moiety in these microenvironments. The glucuronide conjugates of compounds, such as quercetin and luteolin that come from the same polyphenol, functional food family as curcumin, were studied and reported to be capable of deconjugation. In inflammation, β -glucuronidase is known to be released from granulocytes including neutrophils.⁴⁴ When human neutrophils were incubated with ionomycin/cytochalasin B (inflammation inducers), the luteolin glucuronide was converted to free drug. Furthermore, it was demonstrated that deconjugation

was achieved *in vivo* by feeding mice or rats with quercetin or luteolin, and treating (intraperitoneal injection) them with liposaccharides (LPC), which are known to lead to inflammation. The levels of both the free and conjugated form of the drug were tested after sacrificing the animals. It was determined that the plasma and tissue levels of the metabolite were significantly reduced upon the inflammatory response, clearly indicating its deconjugation.^{41, 42}

The levels of curcumin glucuronide captured here in our study are remarkably higher for the low-dose nano-curcumin samples when compared to high-dose unformulated curcumin. This significantly higher bioavailability of the curcumin glucuronide in plasma when using nanoparticles may present a novel therapeutic strategy by employing deconjugation of the metabolite at the specific sites such as inflammation.

4. Conclusions

In summary, we investigated the bioavailability of curcumin and curcumin glucuronide in blood plasma and CNS after oral intake of nano-curcumin and native curcumin in mice. The highly sensitive and validated method of detection clearly distinguished curcumin from its major metabolite, curcumin glucuronide. The results show similar concentrations of curcumin in CNS tissues after oral dosage of nano-curcumin 20 times lower compared to the dosage of unformulated curcumin, which fully support the conclusions drawn by our previous study of curcumin effects of preventing and reversing morphine tolerance and dependence using animal behavioral models. The presence of curcumin in CNS in the first 15 – 30 minutes is essential for suppressing the activity of CaMKII α for the development of opioid tolerance and dependence.

The detected curcumin glucuronide level in blood plasma is significantly higher using the nano-curcumin compared to 20 times higher dose of native curcumin, which may provide a chance to further increase curcumin bioavailability by deconjugation of curcumin glucuronide triggered by codelivery of β -glucuronidase at a specific site such as inflammation.

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Author Contributions

M.S., X.H., P.A.L, P.C. conceived and conducted experiments. M.S., X.H., Z.J.W. and Y.L. interpreted the results. M.S., Y.L. composed the manuscript, with editorial contributions from Z.J.W. All authors have given consent to the final version of the manuscript.

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Figure 1. The process of producing PLGA nanoparticles encapsulating curcumin for oral administration in mice. PLGA-curcumin suspensions are generated via flash nanoprecipitation in the multi inlet vortex mixer (MIVM). The nanoparticles are further freeze dried together with excipients to obtain stable and dried product that can be conveniently resuspended at desired time and concentration for animal dosing.

Figure 2. Representative chromatograms of curcumin extracted from (A) brain and (B) spinal cord. The top one (in red) is the chromatogram of blank tissues. The middle one (in blue) is the chromatogram of curcumin extracted from tissues spiked with curcumin standard (STD). The

bottom one (in green) is the chromatogram of curcumin extracted from tissue samples 15min after oral delivery of low-dose (20mg/kg) nano-curcumin. The analysis uses ion transition for curcumin (367.1>216.9).

Figure 3. The amount of curcumin measured in (A) brain, (B) spinal cord, and (C) plasma after oral administration of low-dose (20mg/kg) nano-curcumin (black symbols and lines), low-dose (20mg/kg) pure curcumin (blue symbols and lines), and high-dose (400mg/kg) pure curcumin (red symbols and lines). All values are reported as the mean $\pm$ S.E.M (n=4).

Figure 4. (A) MS/MS spectrum of curcumin glucuronide showing precursor to product ion transition. (B) The amount of curcumin glucuronide measured in plasma after oral administration of low-dose (20mg/kg) nano-curcumin, low-dose (20mg/kg) pure curcumin, and high-dose (400mg/kg) pure curcumin. All values are reported as the mean $\pm$ S.E.M (n=4). (C) Representative chromatograms for blank plasma, blank plasma spiked with curcumin glucuronide standard, and plasma, brain and spinal cord tissue samples collected 15min after oral delivery of low-dose (20mg/kg) nano-curcumin. The analysis uses ion transition for curcumin glucuronide (543.1>175).

A

B

A

B

C