Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2014 February 15; 0: 70–78. doi:10.1016/j.jchromb.2013.12.039.

A high-throughput quantification method of curcuminoids and curcumin metabolites in human plasma via high-performance liquid chromatography/tandem mass spectrometry

Yu Cao^a, Ronald X. Xu^{b,c}, and Zhongfa Liu^{a,d,*}

^aCollege of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

^bDepartment of Biomedical Engineering, The Ohio State University, Columbus, OH 43210, USA

^cUniversity of Science and Technology of China (USTC) , <addl>Anhui</addl>, Hefei, China

^dComprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

Abstract

Curcuminoids, a mixture of curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC), have shown a variety of clinical benefits for several human chronic diseases including osteoarthritis, rheumatoid arthritis, and type II diabetes. However, the oral bioavailability of curcumin is extremely low due to its avid metabolism to curcumin *O*-glucuronide (COG), curcumin *O*-sulfate (COS), tetrahydrocurcumin (THC), and other minor metabolites. This paper reports a unique liquid chromatography/tandem mass spectrometry (LC-MS/MS) method to quantify curcumin, DMC, BDMC, COG, COS, and THC simultaneously in human plasma. These compounds were extracted with ethyl acetate from human plasma, separated on a BetaBasic-8 column, and monitored on a triple quadrupole mass spectrometer coupled with API electrospray under a negative ion mode. The linearity of these respective curcuminoids and curcumin metabolites was shown in the range of 2–1000 ng/mL with 85–115% accuracy and 20% precision in human plasma. This method was validated according to the US FDA GLP analytic criteria and applied to characterize the pharmacokinetics of curcumin, COG, and COS in human plasma after an oral dose of bioavailable curcumin (nanoemulsion curcumin).

Keywords

Curcuminoids; Liquid chromatography/tandem mass; spectrometry (LC-MS/MS); Curcumin metabolites; Pharmacokinetics

1. Introduction

Curcuminoids, a mixture of curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) are natural polyphenols extracted from the rhizome of turmeric (*Curcuma longa*) [1,2]. Curcuminoids possess a wide range of biological and

pharmacological activities including anti-oxidation, anti-inflammatory, and anti-tumor effects, most of which have been demonstrated from preclinical studies [3]. It is also noticed that the plasma levels of curcuminoids are extremely low (<50 ng/mL) after an oral ingestion of curcuminoids up to 12 g/day [4,5]. This low oral bioavailability of curcuminoids is considered to be due to their poor solubility, poor absorption, and rapid metabolism [6]. Because of the low oral bioavailability, it is very challenging to evaluate the absorption of curcuminoids by monitoring their original forms. Metabolic studies have demonstrated that orally ingested curcumin is extensively transformed to curcumin *O*-glucuronide (COG) and curcumin *O*-sulfate (COS) in both rodents [7,8] and human [5].

Recently, we characterized the mass spectra of curcumin, its two synthetic analogs tetramethyl curcumin (TMC) and dimethylcyclohexyl curcumin (DMCHC), and its two metabolites tetrahydrocurcumin (THC) and curcumin *O*-glucuronide (COG) under a positive ion mode on a Thermo TSQ Quantum triple quadrupole mass spectrometer [9,10]. The protonated molecular ions are the predominant ions in their respective mass spectra; the major fragmentation pathway for curcumin, THC, TMC, and DMCHC is the cleavage of the heptenoid chain between the 3rd and the 4th carbon atoms (Scheme 1) [9]; and the major fragmentation for COG is glycosidic cleavage (Scheme 1) [10]. Two analytic methods for quantification of curcuminoids and COG [10] were established in both mouse and human plasma under a positive mode. These methods were used to characterize their stabilities and pharmacokinetics in mouse and human [9,10]. However, these two methods are incapable of measuring COS. To develop an integrative method for simultaneous quantification of curcumin, DMC, BDMC, COG, COS, and THC, the mass spectra and tandem mass spectra of these curcuminoids and curcumin metabolites under both positive and negative modes were compared. The optimized LC-MS/MS method was validated in human plasma. Its capability to measure the curcuminoids and curcumin metabolites in human plasma was confirmed by sample analysis from healthy human volunteer after an oral administration of nanoemulsion curcumin.

2. Materials and methods

2.1. Reagents and chemicals

Curcumin (>98%, w/w) was purchased from Acros Organics (Morris Plains, NJ) and used without further purification. Curcumin *O*-glucuronide (COG, >95%, w/w) and curcumin *O*-sulfate (COS, >91%, w/w) were custom-synthesized from Cell Mosaic (Worcester, MA). Demethoxycurcumin (DMC, >98%, w/w) and bisdemethoxy-curcumin (BDMC, >98%, w/w) were purchased from Sigma Aldrich. Tetrahydrocurcumin (THC, >94.6%, w/w) were purchased from ChromaDex (Irvine, CA). The internal standard (IS) hesperetin (Hes, >98%, w/w) was obtained as a white powder from the National Cancer Institute (NCI) and used without further purification. The actual concentration of all these standards was justified based on their purity. Analytical HPLC grade acetonitrile, ethyl acetate, methanol, and formic acid were obtained from Fisher Scientific (Waltham, MA). Heparin-treated human plasma was obtained from LAMPIRE Biological Laboratories, Inc. (Pipersville, PA). HPLC grade water (>18 mΩ) was obtained via a Barnstead E-pure water purification system (Dubuque, IA).

2.2. LC-MS/MS analyses

Liquid chromatography was performed on a Shimadzu HPLC system (Shimadzu, Columbia, MD) consisting of an SCL-10A system controller, two LC-10AD pumps, and an SIL-10AD auto-sampler. Curcuminoids (curcumin, DMC, and BDMC), curcumin metabolites (COG, COS, and THC), and internal standard hesperetin were separated on a BetaBasic-8 column (2.1 mm × 50 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA) coupled with a BetaBasic-8 guard column (2.1 mm × 10 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA). The samples were eluted by 50% acetonitrile with 0.1% formic acid at a flow rate of 0.2 mL/min for 5 min.

Curcuminoids, curcumin metabolites, and hesperetin were monitored using an API 3000 mass spectrometer equipped with an electrospray ionization (ESI) quadrupole mass analyzer. Analyst software (Version 1.4.2) was used for system control and data processing. The optimized system parameters were obtained via direct infusion of standard solutions respectively. The ion transition pairs for quantification were selected based on the highest multiple reaction monitoring (MRM) signal under positive (+) mode or negative (−) mode. The levels of curcuminoids and curcumin metabolites were monitored by following ion transitions at *m/z* values of: curcumin 367.4/149.1, DMC 337.3/216.9, BDMC 307.5/186.8, COG 543.7/216.9, COS 447.4/216.9, THC 371.2/235.1, and IS 301.5/163.9 under (−) mode. Under this condition, the needle spray voltage was 4000 V and the heated capillary temperature was 400 °C. The nebulizer gas, curtain gas, and collision gas were tuned to give optimum response. The collision energy was tuned for each analyte individually to obtain an optimum value, 30%, 19%, 30%, 28%, 18%, 25%, and 32% for curcumin, DMC, BDMC, COG, COS, THC, and IS, respectively.

2.3. Sample preparation for calibration standards and quality controls

Stock solutions of curcuminoids and curcumin metabolites were prepared in acetonitrile at 1.0 mg/mL concentrations and stored at −80 °C except COG in methanol. To prepare a calibration curve, an aliquot of 10 μL appropriate diluted standard solution (a mixture of curcumin, DMC, BDMC, COG, COS, and THC, each of 20–10,000 ng/mL) and an aliquot of 10 μL hesperetin (10 μg/mL) were spiked into an aliquot of 100 μL human plasma to yield plasma samples containing 2.0–1000 ng/mL standards, respectively. Quality controls (QCs) were prepared at 2.0, 5.0, 10, 50, and 500 ng/mL concentrations. These samples were diluted with acidified phosphate saline buffer (the pH of PBS was adjusted to 3.2 by formic acid), extracted with 3 mL of ethyl acetate by vortex mixing, and centrifuged. The supernatant was collected and dried under nitrogen gas atmosphere in dark. The residue was then reconstituted in 100 μL of mobile phase (50% acetonitrile with 0.1% formic acid). The reconstituted solution was centrifuged at 13,500 rpm for 4 min and an aliquot of 25 μL supernatant was used for LC-MS/MS analysis.

2.4. LC-MS/MS assay validation

The intra-day validation was determined in six replicates at concentrations of 2.0, 5.0, 10, 50, and 500 ng/mL curcumin, DMC, BDMC, COG, COS, and THC. The inter-day validation was determined across these concentrations in triplicates on three different days. The calibration curves were fitted by a linear regression with a weighting factor of $1/x^2$. The

mean concentrations and the coefficient of variation (CV) of intra-day were calculated as the relative standard deviation (%) from the replicates; the CV of inter-day was calculated as the relative standard deviation (%) of the respective mean concentrations on each individual day for three days. The accuracy of the assay was determined by comparing the corresponding calculated mean concentrations with the nominal concentrations. The lower limit of detection (LLOD) was defined as the lowest concentration with a signal/noise ratio of the analyte peak >3 , and the lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal/noise ratio of the analyte peak >10 .

2.5. Recovery and matrix effect (ME)

A post-extraction spike experiment was performed to evaluate the recovery and matrix effects of curcumin, DMC, BDMC, COG, COS, THC, and the internal standard hesperetin in human plasma. Three separate batches of curcuminoids samples at concentrations of 5.0, 50 and 500 ng/mL, and hesperetin at 1000 ng/mL, were prepared as follows: (a) curcumin, DMC, BDMC, COG, COS, THC, and hesperetin were prepared in 50% acetonitrile with 0.1% formic acid; (b) curcumin, DMC, BDMC, COG, COS, THC, and hesperetin were spiked into the mobile phase reconstituted extract of blank human plasma solution; (c) curcumin, DMC, BDMC, COG, COS, THC, and hesperetin were treated in the same method as for QCs. The recovery of curcumin, DMC, BDMC, COG, COS, and THC was calculated by the ratios of the corresponding peak areas in batch c to those in batch b samples. The IS normalized recovery was calculated by dividing the recovery of the analyte by the recovery of IS. The ME was evaluated by the ratio of the peak areas in batch b to that of batch a samples. The IS normalized ME was calculated by dividing the ME of the analyte by the ME of the IS.

2.6. Stability of curcuminoids and curcumin metabolites in human plasma

To evaluate the two-week stability of curcuminoids in human plasma, standard stock solutions were added to human plasma to yield concentrations of 10, 50, and 500 ng/mL on days 1, 7, and 14. These samples were stored at -80°C immediately. On day 15, all the samples were analyzed using the LC-MS/MS method. A statistical analysis between the freshly prepared samples and the samples after storage was performed by Student's *t*-test ($p < 0.05$).

To evaluate the freeze-thaw stability of curcuminoids, the freshly prepared human plasma samples at these above concentrations were exposed to three sequential freezing and thawing cycles prior to sample analysis. During each cycle, the samples thawed at 4°C for approximately 60 min and were restored at -80°C for the next freezing cycle at 6 h intervals.

To evaluate the post-extraction stability in the reconstituted solution in an auto-sampler (4°C), samples at the concentrations of 50 and 500 ng/mL were subject to LC-MS/MS analysis hourly up to 4 h.

2.7. Analysis of curcumin, DMC, BDMC, COG, COS, and THC in human plasma samples

Two healthy human subjects were given written informed consent before they were involved in this study. The protocol was approved by the OSU Institutional Review Board (protocol No: 2010H0189). A 100 μ L aliquot of human plasma was collected from the healthy volunteers after an oral ingestion of nanoemul-sion curcumin. The plasma was mixed with 10 μ L I.S. solution at a concentration of 10 μ g/mL. The mixture was extracted with ethyl acetate as described in the sample preparation section. An aliquot of 25 μ L reconstituted solution was injected for LC-MS/MS analysis.

3. Results and discussions

3.1. Mass spectrometric analysis of curcumin, DMC, BDMC, COG, COS, and THC under positive and negative modes

The mass spectra of curcumin, DMC, BDMC, COG, COS, THC or Hes were acquired by direct infusion of individual solutions on an API-3000 mass spectrometer coupled with an electrospray ion source under a positive ion mode. The individual solutions were prepared by mixing 10 μ g/mL of curcumin, DMC, BDMC, COG, COS, THC or Hes in 50% acetonitrile containing 0.1% formic acid. Under an suboptimal mass spectrometric parameters, the full scan mass spectra showed prominent protonated molecular ions $[M + H]^+$ of m/z 369.3, 339.4, 309.5, 545.6, 449.4, 373.3, and 303.1 for curcumin, DMC, BDMC, COG, COS, THC, and Hes, respectively. The $[M + H]^+$ ions of the curcuminoids and curcumin metabolites were subjected to collision induced dissociation at an optimized collision energy. The most abundant fragment ions were 177.2, 177.3, 147.2, 369.0, 177.2, 177.2, and 152.6 for curcumin, DMC, BDMC, COG, COS, THC, and Hes, respectively (Fig. 1).

Similarly, their mass spectra were acquired under a negative ion mode. The full scan mass spectra showed predominant deprotonated molecular ions $[M - H]^-$ of m/z 367.4, 337.3, 307.5, 543.7, 447.4, 371.2, and 301.5 for curcumin, DMC, BDMC, COG, COS, THC, and Hes, respectively (Fig. 2A). The $[M - H]^-$ ions of respective curcuminoids were subjected to collision induced dissociation at an optimal collision energy. The most abundant daughter ions were selected for the quantitative ion pairs, which were 149.1, 216.9, 186.8, 216.9, 216.9, 235.1, and 163.9 for curcumin, DMC, BDMC, COG, COS, THC, and Hes, respectively (Fig. 2B). Comparison of the fragment ions of curcuminoids and their metabolites under negative and positive modes demonstrated that the major cleavage sites of curcuminoids and curcumin metabolites are different between (+) and (−) modes on the same mass spectrometer.

To determine which ion mode will offer higher sensitivities for simultaneous quantitation of these curcuminoids and curcumin metabolites, we compared the mass signal intensity of identical concentrations of curcumin, DMC, BDMC, COG, COS, and THC in Q3 between (+) and (−) modes. As shown in Table 1, during direct infusion, $Q3_{\max}$ of COS under (−) mode is more than 100 folds of that under (+) mode; the difference between (+) and (−) mode for the other curcuminoids and the metabolites are within 10 folds. The much higher $Q3_{\max}$ of COS under (−) mode suggests a relatively better detection limit of COS for the

LC-MS/MS method. This assumption was confirmed by a standard curve in the mobile phase generated under both ion modes under suboptimal conditions. Under a positive ion mode, the LLOD is 10 ng/mL (S/N: 3/1) for COS; under a negative ion mode, the LLOD is 1.0 ng/mL (S/N: 6/1) for COS. The LLOD of COS under a negative ion mode was 10 folds of that under a positive ion mode with an even higher S/N ratio. The LLODs (S/N > 5) of curcumin, DMC, BDMC, COG, and THC under positive and negative modes are 1.0 and 1.0, 0.5 and 0.2, 0.5 and 0.2, 1.0 and 1.0, 1.0 and 2.0 ng/mL, respectively. These LLODs under both modes are comparable. Thus, higher sensitivity for COS detection under a negative ion mode leads the LC-MS/MS analysis to be carried out under a negative ion mode.

3.2. LC analyses of curcumin, DMC, BDMC, COG, COS, and THC

We have reported two LC-MS/MS methods for the quantitation of curcumin, THC, TMC, and DMCHC [9] and the quantitation of curcumin and COG [10]. Therefore, we first evaluated the same extraction and LC conditions for these curcuminoids and curcumin metabolites under the negative mode defined from Section 3.1. We have noticed that these curcuminoids and curcumin metabolites were not well separated from each other under these conditions. However, there is no cross-interference of these co-eluting species. Therefore, we maintained the reported column, mobile phase and running time [10]. The curcumin, DMC, BDMC, COG, COS, and THC were separated on a BetaBasic-8 column with an elution of 50% acetonitrile including 0.1% formic acid at a flow rate of 0.2 mL/min. As shown in Fig. 3, the elution peaks for curcumin, DMC, BDMC, COG, and COS were at approximately 3.32, 3.22, 3.15, 1.86, and 2.21 min. There was supposed to be another elution peak for each of them, which was contributed by the keto-enol tautomer. However, at 5 ng/mL level, this peak was too tiny to be observed (Fig. 3). It might be overlapped with the background noises. There were two elution peaks for THC at 2.35 and 3.23 min. The former one was possibly attributed to the diketone form and the latter one was attributed to the enol form as reported previously [9].

3.3. Method validation

All analytes were extracted by ethyl acetate from acidified PBS buffer [10]. Under sub optimal LC/MS-MS conditions, the calibration curves for curcumin, DMC, BDMC, COG, COS, and THC showed the linearity over the concentration range of 2.0–1000 ng/mL with linear regression coefficients greater than 0.993.

The intra-day and inter-day accuracy and reproducibility of these analytes in human plasma were evaluated at concentrations of 2.0, 5.0, 10, 50, and 500 ng/mL. The results were summarized in Table 2. The intra-day and inter-day LLOQs at 2.0 ng/mL for curcumin, DMC, BDMC, COG, COS, and THC were with a CV 20%, accuracy between 85% and 110%, and signal to noise ratio (S/N) greater than 5. All the QCs for the intra-day and inter-day validation showed CVs of less than 15% and accuracy between 85% and 115%. These precision and accuracy values are acceptable according to the FDA criterion of a GLP analytic method validation (Guidance for Industry Bioanalytical Method Validation, p.5, available from the website: <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf>). This confirms that

the current method has a satisfactory accuracy, precision and reproducibility for the simultaneous quantification of all analytes throughout a wide concentration range. No carry-over peaks were observed at the corresponding retention times when blank mobile phase samples were injected after standard samples (curcuminoids, curcumin metabolites, and IS at 1000 ng/mL).

3.4. Matrix effects and recovery

The matrix effect of curcumin, DMC, BDMC, COG, COS, and THC was determined at four QCs of 5.0, 10, 50, and 500 ng/mL while the IS at 1000 ng/mL in triplicate in human plasma. As shown in Table 3, the matrix effects were 69–80%, 69–86%, 67–84%, 43–45%, 71–74%, and 70–79% for curcumin, DMC, BDMC, COG, COS, and THC, respectively. These data suggested that their mass signals were suppressed in the presence of human plasma matrices. This could be due to co-eluent of endogenous plasma components (e.g., fatty acids, triglycerides, and amines) in human plasma. After compensation with the matrix effect of internal standard, the IS normalized ME for all analytes at all tested concentrations are 98–120% except COG approximately 63%. Although the IS normalized ME for COG is relatively low, the small values of CV for all analytes at all tested concentrations suggested the reproducibility of the assay.

The recovery yield of these curcuminoids and curcumin metabolites were also evaluated at the four concentrations in human plasma in triplicates. As shown in Table 3, at concentrations of 5.0, 10, 50, and 500 ng/mL, the recovery yield were approximately 35–45%, 34–45%, 33–43%, 36–41%, 43–44%, and 67–77% for curcumin, DMC, BDMC, COG, COS, and THC, respectively, in a concentration independent manner. It was noticed that the recovery yield of THC was almost twice of the other curcuminoids and metabolites. This observation was consistent with our previous results that THC degraded more slowly when compared to curcumin in cell medium at 37 °C [9]. The higher stability of THC in human plasma indicates that the saturation level of the heptenoid chain plays an important role in the stability of curcuminoids and the metabolites. The more saturated the curcumin skeleton is, the more stable the compound is in human plasma.

3.5. Stability of curcumin, DMC, BDMC, COG, COS, and THC in human plasma

The stability of curcumin, DMC, BDMC, COG, COS, and THC at concentrations of 10, 50, and 500 ng/mL was evaluated in human plasma after storage in a –80 °C freezer for 1, 8, and 14 days. As shown in Table 4, after 14-day storage, 85–115% of the curcuminoids and curcumin metabolites were retained at these test concentrations when compared to freshly prepared samples except COG at 500 ng/mL after 1-day storage and THC at 50 ng/mL after 8- and 14-day storage. However, statistical analysis of their differences demonstrated that these differences were not statistically significant ($P > 0.05$). These results indicated that curcumin, DMC, BDMC, COG, COS, and THC were relatively stable in human plasma over two weeks at –80 °C.

The stability of these analytes after repeated freeze-thaw cycles was also evaluated in human plasma (data not shown). After three freeze-thaw cycles, 93.3%, 104%, and 90.9% of the pre-freezing CUR; 122%, 120%, and 110% of pre-freezing COG; 93.0%, 102%, and 113%

of pre-freezing COS; 89.7%, 103%, and 91.8% of pre-freezing DMC; 87.0%, 96.3%, and 85.5% of pre-freezing BDMC; and 95.0%, 102%, and 98.8% of pre-freezing THC, were found at 10, 50, and 500 ng/mL levels, respectively. These data indicates that curcumin, DMC, BDMC, COG, COS, and THC are relatively stable as a mixture in human plasma over three freeze-thaw cycles.

The stability in the reconstituted solution of human plasma extract in an autosampler (at 4 °C) was also evaluated at 50 and 500 ng/mL (Fig. 4). More than 85% of the extracted curcuminoids, COG and COS remained unchanged over 4 h at the concentrations of 50 and 500 ng/mL, but only 77% of THC remained unchanged at 4 h. This indicates that extracted curcuminoids, COG and COS are quite stable at 4 °C over 4 h, which allows 48 samples analysis in a batch. Since THC is a minor metabolite of curcumin after its oral ingestion and there is no THC detected in human plasma after its oral administration, no further effort were made at this point to achieve higher stability after extraction.

3.6. Quantification of curcumin and COG plasma levels in healthy volunteers after an oral dose

The method described above was applied to measure plasma levels of curcumin, DMC, BDMC, COG, COS, and THC in plasma samples collected from two healthy volunteers (Fig. 5). After an oral administration of nanoemulsion curcumin containing 2 g curcuminoids (85% curcumin, 12% DMC, and 3% BDMC), curcumin reached a C_{max} of 11 ng/mL at 10 min and was detectable up to 1 h; COG reached a C_{max} of 1017 ng/mL at 1 h and was detectable up to 24 h; COS reached a C_{max} of 151 ng/mL at 45 min and was detectable up to 8 h; DMC, BDMC, and THC were below detection limit (2.0 ng/mL).

Conclusions

A simple, rapid, and specific LC-MS/MS method was developed and validated for simultaneous quantification of curcumin, DMC, BDMC, COG, COS, and THC in human plasma. This method provides a comprehensive analytic method to characterize the pharmacokinetics of curcumin, COG, and COS in human plasma after the oral ingestion of curcumin, which will be correlated with pharmacodynamic endpoints in pre-clinical and clinical setting.

Acknowledgments

This work was supported by National Institute of Health (NIH) grants [R21CA135478 (Liu) and R21CA159077 (Liu & Xu)], the National Center for Research Resources [UL1RR025755] and Biomedical Mass Spectrometric Laboratory at The Ohio State University.

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Fig. 1. The tandem mass spectra of the protonated molecular ions of curcumin, DMC, BDMC, COG, COS, and THC under the positive mode.

Fig. 2.

A. The full scan mass spectra of the deprotonated molecular ions of curcumin, DMC, BDMC, COG, COS, and THC under the negative mode. B. The tandem mass spectra of the deprotonated molecular ions of curcumin, DMC, BDMC, COG, COS, and THC under the negative mode. C. The putative fragmentation pathways of curcumin and THC under the negative mode.

Fig. 3.

The subtracted total ion chromatogram (TIC) and subtracted extracted ion chromatograms (XICs) of curcuminoids and curcumin metabolites, and hesperetin in human plasma (5.0 ng/mL of each standard respectively and 1000 ng/mL of hesperetin). (A) TIC; (B) hesperetin (Hes); (C) CUR; (D) COG; (E) COS; (F) THC; (G) DMC; (H) BDMC.

Fig. 4. Stability profile of human plasma spiked with curcuminoids and curcumin metabolites at concentrations of 50 and 500 ng/mL after extraction in an auto-sampler (4°C) for 4 h ($n = 3$). (A) CUR; (B) COG; (C) COS; (D) THC; (E) DMC; (F) BDMC. Data of 50 and 500 ng/mL are indicated by dash line and dash/dots line, respectively.

Fig. 5.

The plasma concentration-time profiles of curcumin, COG, and COS following an oral administration of nanoemulsion curcumin containing 2 g curcuminoids in two healthy volunteers. (A) Curcumin; (B) COG; (C) COS. The concentrations that are not log transformed on the y axis are shown in the left column and the data as a log plot is shown in the right column.

CUR (C₂₁H₂₀O₆, Exact Mass: 368.1260) **DMCHC** (C₂₈H₃₂O₆, Exact Mass: 464.2199) **DMC** (C₂₀H₁₈O₅, Exact Mass: 338.1154) **COG** (C₂₇H₂₈O₁₂, Exact Mass: 544.1581) **BDMC** (C₁₉H₁₆O₄, Exact Mass: 308.1049) **COS** (C₂₁H₂₀O₉S, Exact Mass: 448.0828) **TMC** (C₂₅H₂₈O₆, Exact Mass: 424.1886) **THC** (C₂₁H₂₄O₆, Exact Mass: 372.1573)**Scheme 1.**

The chemical structures, the theoretic exact masses, and the carbon numbering for putative collision-induced fragmentation (shown on CUR) of the curcuminoids (CUR, DMC, and BDMC), curcumin analogs (TMC and DMCHC), and curcumin metabolites (COG, COS, and THC).

Table 1

The signal intensity of the product ion (Q3) of the curcuminoids and the curcumin metabolites during direct infusion under positive and negative modes.

STD	Q3 _{max} (×104 cps)	
	(+)	(-)
CUR	17	48
COG	16	21
COS	0.29	88
DMC	30	150
BDMC	6.7	75
THC	23	150

Table 2

The validation parameters: intra-day and inter-day accuracies and precisions of curcumin, DMC, BDMC, COG, COS, and THC in human plasma.

Standard	Concentration (ng/mL)	Intra-day Mean	CV (%)	Accuracy (%)	Inter-day Mean	CV (%)	Accuracy (%)
CUR	2.0	1.97	5.41	98.7	2.01	4.71	101
	5.0	4.86	6.95	97.2	4.90	10.9	97.9
	10	10.1	7.62	101	9.86	1.78	98.6
	50	50.0	6.67	100	51.2	2.70	102
	500	541	4.65	108	520	7.69	104
COG	2.0	2.06	6.30	103	1.94	12.4	96.8
	5.0	4.32	14.0	86.3	4.51	6.54	90.2
	10	9.57	14.8	95.7	9.31	2.52	93.1
	50	52.6	7.84	105	50.7	5.01	101
	500	484	5.88	96.9	500	7.99	100
COS	2.0	1.75	20.0	87.4	1.70	2.66	84.9
	5.0	4.41	6.24	88.3	4.96	7.85	99.2
	10	9.73	7.23	97.3	10.3	5.12	103
	50	53.1	4.26	106	55.4	4.14	111
	500	572	5.24	115	538	8.24	108
DMC	2.0	2.08	5.90	104	1.89	8.45	94.7
	5.0	4.76	6.41	95.1	4.88	9.99	97.6
	10	10.1	8.59	101	10.1	0.58	101
	50	50.2	5.80	100	52.3	3.43	105
	500	537	4.73	107	520	7.56	104
BDMC	2.0	2.17	3.13	108	1.86	13.5	92.9
	5.0	4.75	8.00	94.9	5.21	9.18	104
	10	10.3	5.20	103	10.2	3.27	101
	50	48.2	6.46	96.3	49.9	3.49	100
	500	533	7.01	107	543	2.76	109
THC	2.0	2.10	5.89	105	2.19	3.57	110
	5.0	5.46	4.62	109	5.26	3.94	105
	10	9.79	4.80	97.9	10.3	14.0	103

Standard	Concentration (ng/mL)	Intra-day Mean	CV (%)	Accuracy (%)	Inter-day Mean	CV (%)	Accuracy (%)
	50	44.0	4.66	87.9	49.0	14.5	98.1
	500	431	4.60	86.3	461	6.79	92.2

ND: not determined.

Table 3

The validation parameters: recovery and matrix effect of curcumin, DMC, BDMC, COG, COS, THC, and IS in human plasma, and IS normalized recovery and matrix effect of all analytes.

Standard	Concentration (ng/mL)	Recovery (%)	Matrix effect (%)	IS-Recovery (%)		IS-ME (%)	
				Mean	CV (%)	Mean	CV (%)
CUR	2.0	ND	ND	ND	ND	ND	ND
	5.0	35.2	78.2	40.8	7.70	109	6.08
	10	40.2	69.2	48.2	4.04	99.3	8.72
	50	36.8	70.5	44.0	8.84	104	8.54
	500	44.6	80.3	55.4	3.37	111	2.51
COG	2.0	ND	ND	ND	ND	ND	ND
	5.0	39.4	44.9	46.5	17.8	63.4	13.0
	10	35.5	44.3	42.7	4.59	63.5	7.11
	50	40.0	42.5	47.4	5.02	62.3	7.70
	500	40.8	45.4	50.8	7.58	62.7	2.78
COS	2.0	ND	ND	ND	ND	ND	ND
	5.0	44.2	74.1	51.2	7.07	112	16.7
	10	44.4	74.3	53.4	3.75	108	7.93
	50	43.7	70.5	52.2	4.11	104	9.21
	500	43.1	74.4	53.6	3.61	103	2.43
DMC	2.0	ND	ND	ND	ND	ND	ND
	5.0	33.5	79.4	38.9	6.49	111	4.34
	10	37.7	70.7	45.3	1.86	102	11.4
	50	35.9	68.9	43.0	7.60	101	2.64
	500	45.0	85.8	56.0	5.06	119	1.14
BDMC	2.0	ND	ND	ND	ND	ND	ND
	5.0	32.5	81.8	37.8	9.00	114	8.78
	10	38.4	69.2	46.2	4.05	99.7	12.3
	50	35.6	67.1	42.6	6.64	98.5	5.66
	500	42.5	83.8	53.1	8.88	116	1.74
THC	2.0	ND	ND	ND	ND	ND	ND

Standard	Concentration (ng/mL)	Recovery (%)	Matrix effect (%)	IS- Recovery (%)		IS-ME (%)	
				Mean	CV (%)	Mean	CV (%)
Hesperetin	5.0	73.5	78.6	85.0	8.97	110	8.85
	10	70.2	70.9	84.6	2.52	102	11.9
	50	66.6	70.1	79.8	7.47	103	8.18
	500	77.0	78.7	95.7	3.97	109	5.07
	1000	83.5	70.5	N/A	N/A	N/A	N/A

ND: not determined.

N/A: not available.

Table 4

The stabilities of curcumin, DMC, BDMC, COG and COS in human plasma at -80°C . Two-tail Student's *t*-test analysis showed that the difference is statistically insignificant ($p > 0.05$).

STD	Concentration (ng/mL)	1-day storage	8-day storage	14-day storage
CUR	10	89.9 ± 5.3	91.8 ± 5.9	99.3 ± 15
	50	93.1 ± 8.4	102 ± 12	88.7 ± 1.7
	500	103 ± 13	95.5 ± 4.3	104 ± 10
COG	10	102 ± 11	105 ± 2.6	112 ± 2.9
	50	106 ± 13	116 ± 3.3	112 ± 6.0
	500	83.9 ± 4.0	116 ± 6.3	112 ± 18
COS	10	111 ± 11	97.4 ± 13	105 ± 1
	50	104 ± 0.44	97.4 ± 3.6	104 ± 1.7
	500	108 ± 12	112 ± 4.5	113 ± 11
DMC	10	92.1 ± 11	99.5 ± 9.6	97.6 ± 8.9
	50	91.0 ± 4.5	99.4 ± 8.5	93.1 ± 8.1
	500	105 ± 11	100 ± 6.1	107 ± 5.6
BDMC	10	90.3 ± 3.8	96.6 ± 8.2	93.6 ± 13
	50	86.6 ± 4.1	94.1 ± 4.7	101 ± 4.4
	500	102 ± 5.5	102 ± 5.3	115 ± 14
THC	10	96.1 ± 15	92.9 ± 5.2	103 ± 12
	50	98.9 ± 3.6	78.6 ± 11	74.4 ± 14
	500	106 ± 0.34	114 ± 7.1	106 ± 21