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A liquid chromatography–tandem mass spectrometric method for quantification of curcuminoids in cell medium and mouse plasma

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ABSTRACT

Curcumin and tetrahydrocurcumin (THC) have been found as potent DNMT1 inhibitors, but they suffer from low oral bioavailability and rapid metabolism *in vivo*. To circumvent these problems, two curcumin analogs: 1,7-bis(3,4-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadiene-3,5-dione (TMC) and 1,7-bis(3,4-dimethoxyphenyl)-4-cyclohexyl-1,6-heptadiene-3,5-dione (DMCHC) have been synthesized to enhance their stability by blocking the two metabolic sites, the phenolic and C4 methylene moieties. Both compounds have shown inhibitory activity on M. SssI similar to that of curcumin and THC (Poster, M1114, AAPS, 2009). Preclinical pharmacokinetics has yet to be performed. In this paper, a simple liquid chromatography–tandem mass spectrometric method was developed for the determination of these four curcuminoids in cell medium and mouse plasma. The method showed linearity from 1 to 1000 ng/mL with the lower limit of quantification of 1 ng/mL in cell medium, and 5 ng/mL in mouse plasma for all test curcuminoids. The within-day coefficients of variation were found to be below 15% and the accuracy was in the range of 85–115%. This method was subsequently used to evaluate their stability in these matrices and a pilot pharmacokinetics of curcumin, DMCHC and TMC in mice after an intraperitoneal (i.p.) cassette dosing of 10 mg/kg each. Curcuminoids degraded in two phases with terminal half lives of 186, 813, 724, and 2000 min for curcumin, THC, TMC, and DMCHC, respectively, in cell culture medium. In plasma, their respective half lives were 111, 232, 1202 and 3000 min. These data demonstrated that their stability is in the order curcumin < THC < TMC < DMCHC in both matrices. Following an i.p. cassette dose, both TMC and DMCHC showed the prolonged elimination half life (1.0, 1.0 h, respectively vs 0.4 h for curcumin) and an increased drug exposure as described by the area under the curve (0.64, 0.98 μM h, respectively vs 0.4 μM h for curcumin).

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1. Introduction

Curcumin, a major constituent of turmeric (*Curcuma longa* L.), has been valued worldwide as a potential food additive, coloring agent and spice, and more importantly showing pharmacological activities such as anti-inflammatory, antimicrobial, antioxidant, anti-parasitic, anti-mutagenic and anti-cancer activities [1–4]. Due to its potential inhibition of mutagenesis and chemically induced carcinogenesis via perturbation of multiple molecular targets during these processes, it has become a vital lead molecule for the development of various potential anti-cancer agents that can be used for the prevention and/or treatment of various cancers [5–8].

In the course of discovery and development of novel DNA methylation inhibitors from natural sources, curcumin, its two natural analogs: demethoxycurcumin and bisdemethoxycurcumin, and one of its reduced metabolites tetrahydrocurcumin (THC) have been found as potent DNMT1 inhibitors and a pilot structure–activity relationship study demonstrated that the beta-diketone is an essential pharmacophore of curcuminoids for inhibition of M. SssI [9]. However, they suffer from low oral bioavailability and rapid *in vivo* metabolism. To circumvent these problems, two curcumin analogs: 1,7-bis (3,4-dimethoxyphenyl)-4,4-dimethyl-1,6-heptadiene-3,5-dione (TMC) and 1,7-bis(3,4-dimethoxyphenyl)-4-cyclohexyl-1,6-heptadiene-3,5-dione (DMCHC) have been synthesized to enhance their stability by blocking the two metabolic sites, the phenolic and C4 methylene moieties. Both compounds have shown inhibitory activ-

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ity on M. SssI similar to that of curcumin and THC (Poster, M1114, AAPS, 2009). Preclinical pharmacokinetics is yet to be performed.

Several methods based on high performance liquid chromatography (HPLC) [10–12], capillary electrophoresis [13], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [14] and matrix assisted laser desorption/ionization (MALDI) [15] were reported for quantification of curcumin and its analogs from turmeric powder, food products, rat plasma, and other biological matrices. Recently, a simple and useful method for the analysis of curcumin analogs from rat plasma samples using solvent extraction and negative mode LC–MS/MS was reported [16], and this method can be efficiently used for the rapid screening of curcumin and THC [16]. However, due to conversion of 4-OH group to 4-methoxy group in TMC and DMCHC and the introduction of the hydrophobic moieties in TMC and DMCHC, it is expected that their mass signal in negative mode will be attenuated and their interaction with the surface of a reverse phase C18 column is stronger and results in a rather long retention time, therefore, in this paper, we report a sensitive and rapid LC–MS/MS method for simultaneous determination of these four curcuminoids in cell medium and mouse plasma on a C8 column under a positive mode, and its application in their stability in these matrices and a pilot plasma pharmacokinetic study of curcuminoids in mice.

2. Experimental

2.1. Reagents and chemicals

Curcumin (95%) was purchased from Acros Chemicals (USA) and is used as such without any further purification. THC, TMC, DMCHC were synthesized and purified as per the respective reported methodologies [17]. Their structures and purities were determined by HPLC, NMR and mass spectrometric methods. All the chemicals were more than 95% pure. Analytical HPLC grade methanol, acetonitrile, ethyl acetate and formic acid were obtained from Fisher Scientific (Waltham, MA, USA). The internal standard hesperetin was obtained from the National Cancer Institute NCI (Bethesda, MD, USA). The heparin-treated mouse plasma was obtained from LAMPIRE Biological Laboratories, Inc. (Pipersville, PA, USA,). The RPMI cell medium used for the cell culture was obtained from Invitrogen Corporation (Carlsbad, CA, USA). A Barnstead E-pure water purification system (Dubuque, IA) was used to obtain HPLC grade water (>18 mΩ).

2.2. Calibration standards and quality controls preparation and extraction procedure

Stock solutions (1 mg/mL) of curcumin, THC, TMC and DMCHC were prepared in acetonitrile (ACN) and stored in −80 °C. Standard solutions with concentration ranging from 10 to 10,000 ng/mL were prepared by further dilution from the stock solutions with acetonitrile. For calibration curve, 20 μL of the appropriate diluted standard solutions were spiked either into 200 μL RPMI cell medium and 100 μL mouse plasma which contains constant amount of hesperetin at a final concentration of 1000 ng/mL. Quality controls (QC) were prepared at 1.0, 5.0, 50 and 500 ng/mL. These samples were extracted with 1 mL of ethyl acetate for 60 min by mechanical shaking. The samples after extraction were centrifuged at 11,000 × *g* for 2 min and were placed on dry ice for 1 min. The supernatant ethyl acetate was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of 50% acetonitrile containing 0.1% formic acid. The reconstituted solution was centrifuged at 11,000 × *g* for 2 min and an aliquot 20 μL supernatant solution was used for analysis.

2.3. Preparation of samples for stability studies

The curcuminoids were added to 2 mL of RPMI cell medium containing 10% fetal bovine serum and mouse plasma at a concentration of 1000 ng/mL. The solutions were incubated at 37 °C in a water bath for 24 h. An aliquot of 200 μL was drawn at the following time points: 0, 10, 20, 40, 60 min, 2, 4, 6, 8 and 24 h from this incubation pool. The samples were frozen immediately after drawing from the incubated samples in a −80 °C freezer till the time of analysis.

2.4. Recovery and matrix effects

The matrix effects were quantitatively measured by extracting 100 μL each of cell medium and mouse plasma with 1 mL ethyl acetate as described in Section 2.2. The residues after evaporation were reconstituted with 100 μL of standard solution containing each curcuminoid at concentrations of 1, 5, 50 and 500 ng/mL and internal standard at 1000 ng/mL. Samples were prepared in triplicates and analyzed along with the standard solutions prepared in the mobile phase. The difference in signal intensity was measured by calculating the peak area ratios of curcuminoids from the matrix and the mobile phase samples.

2.5. LC–MS/MS analysis

The LC–MS/MS analysis was carried out in positive ion ESI mode on a Thermo TSQ Quantum triple quadrupole mass spectrometer connected to a Shimadzu 20 ADVP pump, SIL 20A auto sampler and CBM 20 A controller. The analysis was conducted on a Beta basic C8 column (2.1 mm × 50 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA) coupled with a Beta basic C8 Javelin guard column (2.1 mm × 10 mm, 2 μm, Thermo Hypersil-Keystone, Bellefonte, PA). The mobile phase 50% acetonitrile containing 0.1% formic acid was used in isocratic mode at a flow rate of 0.2 mL/min. The total run time of the analysis for the present method was 12 min. The sheath gas and auxillary gas were tuned to give optimum response as necessary. The needle voltage was 3.5 kV. Argon was used as collision gas at collision energy of 20–30%. The collision energy is tuned for each analyte individually to obtain an optimum value. The analytes were quantified using selected ion reaction monitoring (SRM). The ion transitions m/z 369 → 177, m/z 373 → 137, m/z 425 → 191, m/z 465 → 191 and m/z 303 → 153 at the respective collision energies 32, 25, 29, 32 and 30% were used for the determination of curcumin, THC, TMC, DMCHC, and hesperetin, respectively.

The structures of these curcuminoids and their putative fragments are also confirmed through high resolution mass spectrometry (HRMS) by measuring their accurate mass. The HRMS analysis was carried out on a Micromass Q-TOF micro quadrupole-time of flight hybrid mass spectrometer (Waters Inc., Bellefonte, USA) equipped with a lock-spray ionization source. A respective solution of these curcuminoids (10 μg/mL) in 50% acetonitrile containing 0.1% formic acid were introduced into the mass spectrometer by direct infusion through the electrospray probe at 10 μL/min. The calibration standards 10 μg/mL polyethyleneglycol (PEG 300 + PEG 600) prepared in 2 mM ammonium acetate in 50% acetonitrile and 10 μg/mL NaI-CsI in 50% 2-propanol, were introduced through the lock-spray source at the interval of every 5 s for 1 min. The electrospray and lock spray probes were operated in positive ion mode with 3.2 kV spray voltage. Nitrogen was used as cone gas and desolvation gas at 50 L/h and 400 L/h, respectively. The HRMS of fragment ions of the analytes was obtained using the in-source fragmentation technique by slightly varying in the extraction voltage between 30 and 60 V.

2.6. The pharmacokinetics of cassette bolus i.p. dosing of curcumin, DMCHC, and TMC in mice

CD2F1 mice (~20 g) (Harlan, Indianapolis, IN) were used in this study. All animal procedures were performed according to a protocol in compliance with The Ohio State University Laboratory Animal Resources (ULAR) policies, which adhered to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Curcumin, DMCHC, TMC formulated in DMSO, at a dose of 10 mg/kg in a 100 μL aliquot [within the oral LD50 of DMSO (7.9 g/kg)] was i.p. administrated to mice. The blood was removed by cardiac puncture under CO_2 anesthesia at the following time schedule of 0 (pre-dose), 5, 10, 20, 30, 60, 120, and 240 min after dosing was collected for the full-set of pharmacokinetics study (n = 3). The blood samples were centrifuged at 1000 g for 5 min in a 4 °C micro-centrifuge and the supernatant of each was collected and kept at −80 °C until analysis. The curcumin levels in plasma were measured using the aforementioned LC–MS/MS assay. Plasma concentration–time data were analyzed by the WinNonlin computer software (Pharsight 5.0, Mountain View, CA) using appropriate pharmacokinetic models.

2.7. Analysis of curcumin and TMC in mouse plasma

An aliquot of 100 μL of PK plasma samples of curcumin and TMC was mixed with 10 μL I.S (10 μg/mL stock in 50% acetonitrile). The resulting solution was extracted and processed according to the protocol described in Section 2.2. An aliquot of 10 μL of the final solution was injected into LC for LC/MS/MS analysis.

3. Results and discussion

Curcumin has shown significant anti-cancer activities in various rodent models [1,3,4,6,7,18–20]. It can form complexes with metal ions, e.g. cupuric [21] ion due to the presence of the ortho methoxy, phenolic and diketo groups in the molecule, which has been proposed to be one of the possible mechanisms for its instability when it is added to the solutions containing metal ions like PBS, cell medium, and plasma. Also, curcumin contains two active double bonds, which are active towards nucleophilic functional groups, e.g. thiol group of various plasma proteins to form curcumin–protein conjugates [22,23]. It also contains an active methylene moiety, which can be easily oxidized in the presence of free radical and oxygen. In addition to their contribution to curcumin stability, these active groups have been proposed to play a very important role in the metabolism, pharmacokinetics and biological activity of curcumin in various biological systems. Hence, to evaluate the contribution of these functional groups to the stability of curcumin and their pharmacological activities, we synthesized THC, in which the double bonds of curcumin are reduced by hydrogenation; TMC, in which active phenol groups and the active methylene carbon at the fourth position of the heptenoid chain are deactivated by methylation, and a more sterically hindered methylated curcumin, DMCHC by the addition of a spiro cyclohexyl ring at the fourth carbon atom. To evaluate their relative stability, a rapid and sensitive method was developed for the simultaneous determination of these curcuminoids in cell medium and mouse plasma followed by its application in their stability study in these matrices, and a pilot PK study of curcumin and TMC in mice.

3.1. Mass spectrometric analysis of curcuminoids

The standard solutions of 10 μg/mL respective curcuminoids in 50% acetonitrile containing 0.1% formic acid were infused directly into the mass spectrometer ESI source under a positive ion mode. The observed full scan mass spectra showed prominent protonated molecular ions [MH^+] ions of *m/z* 369, 373, 425 and 465 for curcumin, THC, TMC and DMCHC, respectively. The MH^+ ions of each curcuminoid were subjected to collision induced dissociation at average collision energy of 30%. The molecules undergo significant fragmentation to yield the following fragment ions of *m/z* 177, 137, 191 and 191 for their respective MH^+ ions as shown in Fig. 1. Curcumin, TMC and DMCHC showed a fragment ion formed from the cleavage of heptenoid chain between 3rd and 4th carbon atoms (between the carbonyl and methylene groups, shown in Scheme 1) whereas THC showed a fragment ion as a stable tropilium cation generated from the cleavage of the first and second carbon atoms of the heptenoid chain. The collision energies were optimized for each curcuminoid to obtain the most intense fragment ion. Based on their mass spectra and tandem mass spectra, the following transitional channels: *m/z* 369 → 177, *m/z* 373 → 137, *m/z* 425 → 191, and *m/z* 465 → 191 were selected for monitoring curcumin, THC, TMC and DMCHC, respectively. The structures of these curcuminoids and their fragments were also confirmed by their accurate mass. The differences in the theoretical accurate masses and the experimentally measured ones are below 5 ppm, which suggest that the proposed fragment structures are reasonable. The molecular formulae and possible fragmentation pathway are shown in Scheme 1.

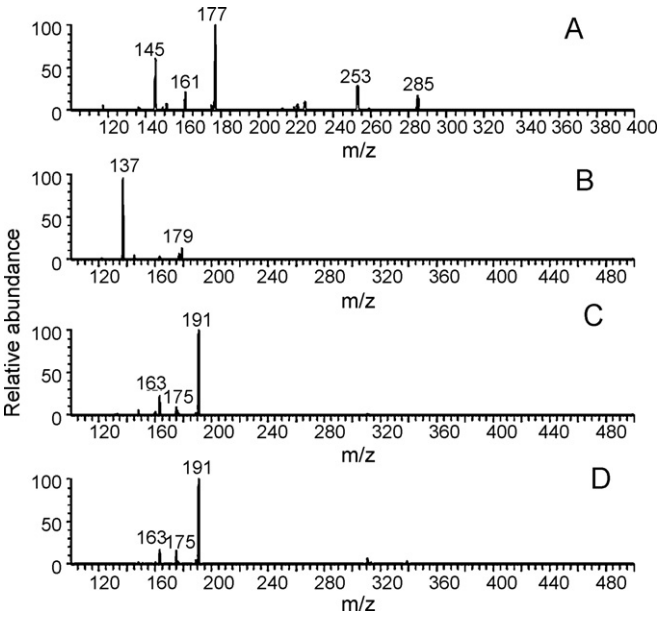
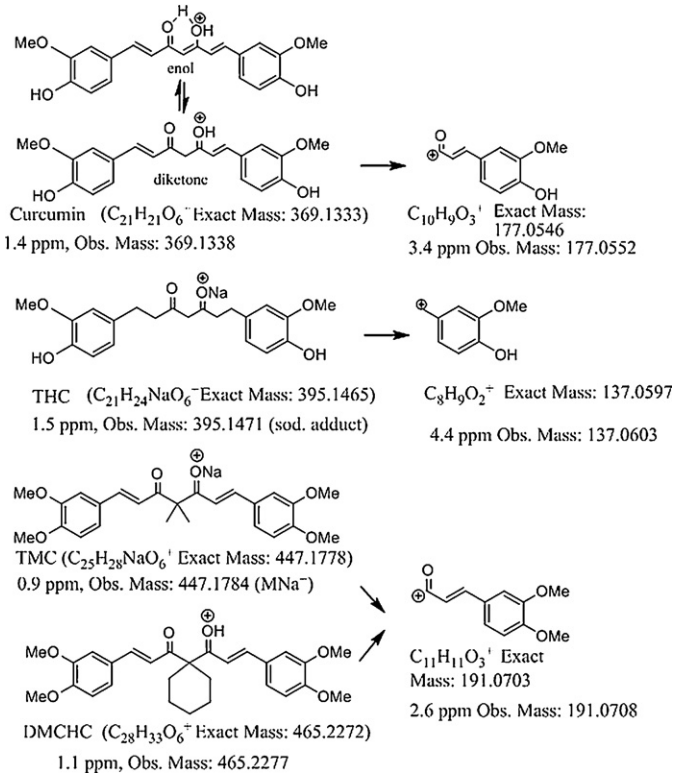


Fig. 1. The tandem mass spectra of the protonated molecular ions of curcumin with *m/z* 369 (A), tetrahydrocurcumin with *m/z* 373 (B), TMC with *m/z* 425 (C), and DMCHC with *m/z* 465 (D).

3.2. Liquid chromatographic separation of curcumins

Compared to curcumin and THC, TMC and DMCHC are much more hydrophobic. Therefore, the present method is developed on a C8 analytical column in order to reduce the hydrophobic interactions so that the analytes are eluted in a shorter period of time. As shown in Fig. 2, there is one peak at 1.82 min in the extracted ion chromatogram (XIC) of the internal standard hesperetin, and there are two peaks in XICs of curcumin at 1.61 and 2.82 min (2B), THC at 1.87 and 2.73 min (2C) and DMCHC at 8.82 and 9.64 min (2E), and there is a shoulder peak along with a big peak at 4.26 min in the XIC of TMC. The retention time is consistent with their hydrophobicity. The two peaks in the XICs of curcumin and THC are attributed to their two tautomers, the diketone and keton-enol tautomeric forms as shown in Scheme 1 [24]. Although curcumin and THC are



Scheme 1. The chemical structures and the putative collision-induced fragmentation pathways and the observed high resolution masses, the theoretic exact masses and the molecular formulae of curcumin (MH^+), tetrahydrocurcumin (MNa^+), TMC (MH^+) and DMCHC (MNa^+) and their putative fragments.

eluted at similar time, their mass chromatograms could be clearly distinguishable as they have different molecular masses and fragmentation. The two peaks in the XIC of DMCHC and the shoulder peak along with a big peak in the XIC of TMS may indicate that DMCHC and TMC may exist in two isomeric rotators due to the potential hindrance of substituted C4 in these two molecules. As these peaks represent different forms of these curcuminoids, the total peak areas of these two peaks in their respective XICs were used for calculations. The extracted ion chromatograms of 2 ng/mL curcumin and its analogs in mouse plasma are shown in Fig. 2. The figure also indicated that there is no significant interference with the analytes from the plasma matrix indicating the specificity of the method.

3.3. Method development and validation

Ethyl acetate was used to extract them from these matrices. The linearity of the method was tested over the concentration range of 1.0–1000 ng/mL for these curcuminoids. Good linearity was obtained for curcuminoids with a linear regression coefficient greater than 0.99. The method was validated at 1, 5, 50 and 500 ng/mL concentrations with six replicates of samples and the data showed acceptable within-day precision and accuracy values according to the FDA criterion of an GLP analytic method validation (Guidance for Industry Bioanalytical Method Validation, p.5, available from the website: http://www.fda.gov/cder/guidance/4252fnl.pdf). The matrix effect and extraction recoveries were evaluated at the concentration of 1, 5, 50 and 500 ng/mL for all curcuminoids in each matrix. The matrix effects were quantitatively compared to their solutions in the mobile phase. The results showed that the mass signal of curcuminoids were not inhibited very much by the matrices and more than 85% of their mass signal could be obtained except in the case curcumin in mouse plasma possibly due to its instability. Similarly, the recoveries were above 85% for all curcuminoids from the matrices except for curcumin in cell medium and mouse plasma which was only 39.1 ± 12.2%. The regression equations, intra-day and inter-day accuracies along with percentage RSD values for mouse plasma and cell medium are presented in Tables 1 and 2. Some deviations were observed in the low concentration range and the LLOQ value of curcumin was 5 ng/mL with a CV of 20%. Since matrix effects of other curcuminoids are not very significant and

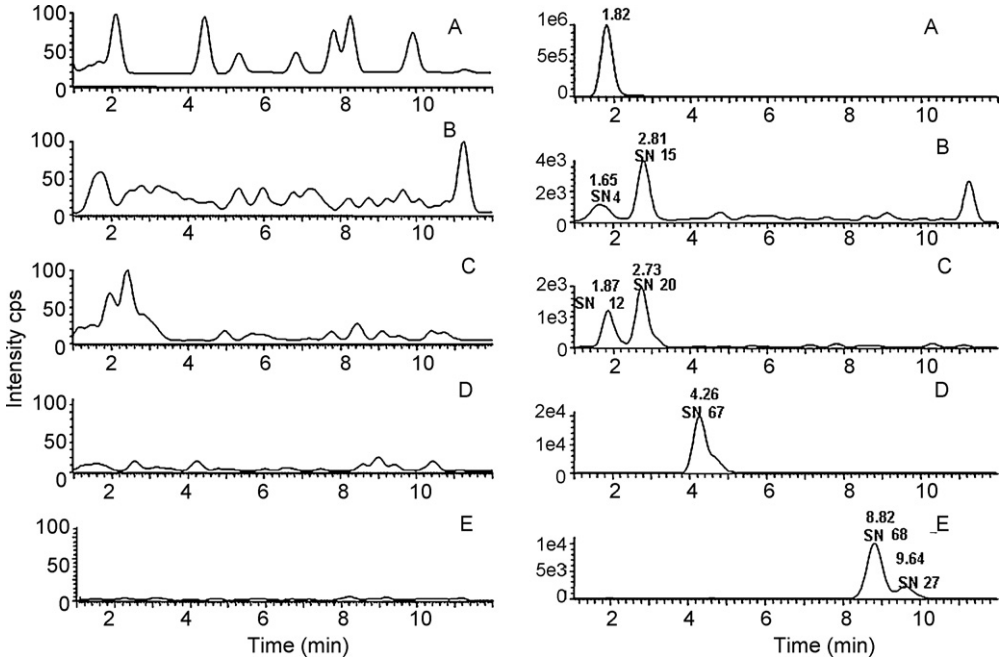


Fig. 2. The extracted ion chromatograms of curcuminoids: hesperetin (A), curcumin (B), THC (C), TMC (D), and DMCHC (E), obtained from the LC–ESI-MS/MS analysis of extraction residue of mouse plasma spiked with 2 ng/mL (right) compared to the corresponding chromatogram (left) from that of blank mouse plasma defining their specificity in mouse plasma.

Table 1
The regression equation and intra- and inter-day validation parameter results of these curcuminoids in mouse plasma.

Analyte	Recovery%	Regression equation	Intra-day validation Accuracy (CV) %			Inter-day validation Accuracy (CV) %		
			5 ng/mL	50 ng/mL	500 ng/mL	5 ng/mL	50 ng/mL	500 ng/mL
Curcumin	39.5 ± 15.8	$Y = 0.0014X + 0.0035$ $R^2 = 0.9994$	103.1 (14.01)	100.8 (3.14)	99.7 (6.22)	92.7 (7.61)	98.5 (3.56)	98.5 (5.02)
THC	100.9 ± 0.9	$Y = 0.0016X + 0.0174$ $R^2 = 0.9979$	90.3 (5.64)	100.2 (3.25)	109.9 (5.92)	89.9 (5.36)	100.4 (1.94)	105.5 (7.51)
TMC	104.4 ± 8.0	$Y = 0.0107X + 0.0763$ $R^2 = 0.9993$	86.2 (9.23)	98.9 (9.72)	99.5 (7.87)	88.5 (15.10)	95.0 (7.89)	100.1 (3.99)
DMCHC	95.4 ± 3.6	$Y = 0.0082X - 0.0012$ $R^2 = 0.9991$	97.9 (9.35)	98.8 (6.24)	101.2 (5.96)	95.64 (9.25)	100.5 (5.78)	97.5 (7.21)

hence the rest of them were determined up to a concentration of 1 ng/mL. A small interfering peak was observed for THC in the blank plasma, whose peak intensity was lower than that of 1 ng/mL concentration. Hence, the peak area of the peak obtained in the blank plasma was constantly subtracted from the total peak area of the THC peaks throughout the analysis. Therefore, the method is suitable for quantitative determination of these curcuminoids in these matrices and may extend to other biological matrices.

3.4. Stability of curcuminoids in the matrices

It is very important to know the stability of curcuminoids in the respective matrices in order to ascertain their stabilities and availability of the chemicals during the period of treatment. Therefore evaluation of their stability in matrices cell medium and mouse plasma will reveal whether THC, TMC and DMCHC can be used as stable hypomethylation agents to outfit the potential pharmacological barrier of curcumin as hypomethylation agents. Therefore, the stability at a concentration of 1000 ng/mL of these respective curcuminoids in each matrix at the following time points 0, 10, 20, 40, 60 min, 2, 4, 6, 8 and 24 h at 37 °C was studied using the above method. The results were detailed as follows.

In cell medium, the observed data showed that curcumin is stable in 1 h and then started degrading gradually. Almost 50% and 90% of curcumin is degraded by 3 and 8 h, respectively, and the elimination half life is 186 min. It could not be detected after 24 h. These data demonstrated that it is not a single phase, but two phase degradation of curcumin in cell medium. THC and TMC have higher stability and slower degradation rate when compared to curcumin with half lives of 813 and 724 min, respectively. DMCHC has higher stability when compared to all other tested curcuminoids with a half life of 2000 min. The data indicate that the stabilities of curcuminoids are in the order curcumin < THC < TMC < DMCHC. The time–concentration curves describing the stability of these curcuminoids in the cell medium are shown in Fig. 3A.

In mouse plasma, the stability data indicated that curcumin is rapidly decomposed more than 90% by 2 h and is almost below the detection levels by 4 h. THC is also gradually decreased, but not as rapidly as curcumin. Almost 50% of THC was degraded by 4 h and 90% by 8 h and below the detection limit after 24 h, indicating complete degradation. TMC, and DMCHC also showed a slow degradation, but much more stable compared to curcumin and THC. The half lives of TMC and DMCHC in mouse plasma are much longer than that of curcumin and THC. The half life data of these curcuminoids was calculated as 111, 232, 1202 and 3000 min for curcumin,

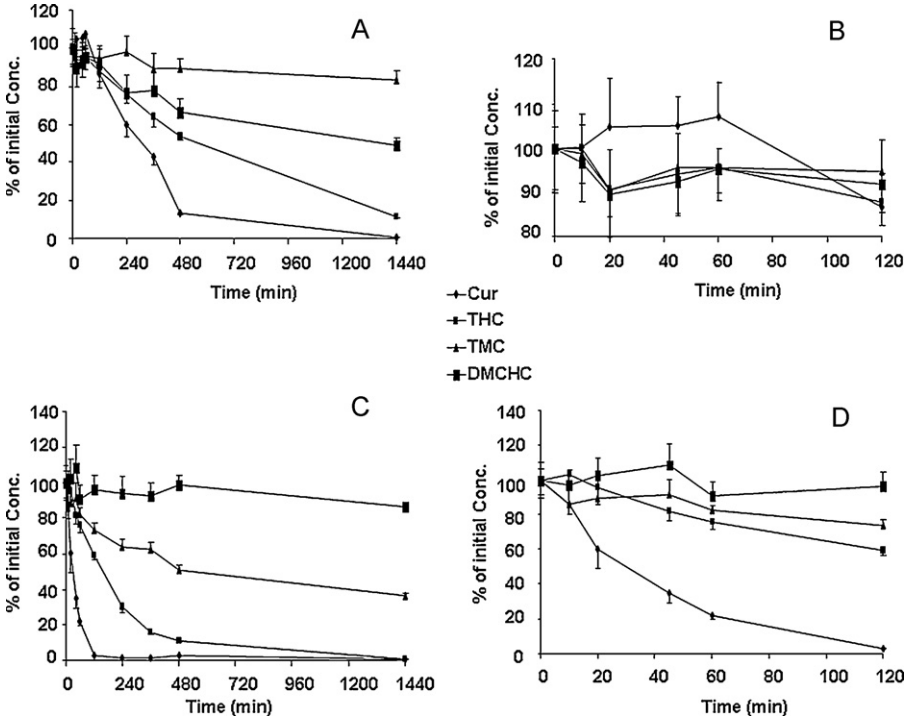


Fig. 3. The time–recovery percentage profile of curcuminoids in (A) cell medium (zoom in from 0 to 120 min [B]) and (C) mouse plasma (zoom in from 0 to 120 min [D]) at 37 °C for 24 h.

Table 2
The regression equation and intra- and inter-day validation parameter results of these curcuminoids in RPMI cell culture medium.

Analyte	Recovery%	Regression equation	Intra-day validation		Accuracy (CV) %		Inter-day validation		Accuracy (CV) %	
			1 ng/mL	5 ng/mL	50 ng/mL	500 ng/mL	1 ng/mL	5 ng/mL	50 ng/mL	500 ng/mL
Curcumin	39.1 ± 12.2	$Y = 0.0034X + 0.0039$ $R^2 = 0.9992$	103.5 (4.85)	98.64 (11.61)	96.56 (0.93)	97.02 (14.01)	106.02 (2.87)	100.9 (2.07)	96.02 (2.24)	105.55 (7.23)
THC	92.1 ± 4.7	$Y = 0.0018X + 0.0003$ $R^2 = 0.9998$	86.8 (7.33)	93.52 (5.22)	101.15 (3.59)	102.37 (3.67)	110.91 (13.9)	109.17 (11.17)	97.70 (3.05)	100.18 (3.78)
TMC	85.8 ± 2.4	$Y = 0.0097X - 0.0254$ $R^2 = 0.9993$	98.25 (5.14)	98.41 (9.32)	97.52 (10.65)	99.92 (4.8)	102.48 (5.5)	107.74 (7.94)	98.6 (1.19)	99.9 (1.14)
DMCHC	86.0 ± 8.4	$Y = 0.0058X - 0.0453$ $R^2 = 0.999$	97.26 (10.59)	96.16 (8.98)	100.95 (7.53)	101.08 (12.8)	98.82 (14.3)	94.9 (11.25)	103.16 (17.03)	97.01 (6.52)

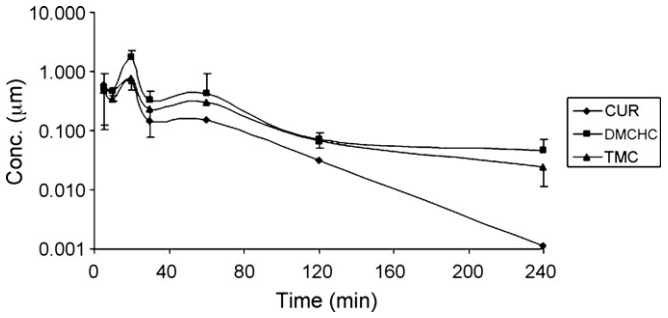


Fig. 4. The plasma concentration–time profile of curcumin, DMCHC, TMC following a cassette i.p. administration of curcumin, DMCHC, TMC at 10 mg/kg, respectively ($n = 3$).

THC, TMC and DMCHC, respectively and their time–concentration curves describing their stability are shown in Fig. 3B.

The stability order of these curcuminoids in both cell medium and mouse plasma is consistent with their structural optimization. Curcumin has less stability due to the presence of the active phenolic groups (4-OH), double bonds (C=C–CO), and C4 methylene group between two carbonyl groups (CH_2). The increased stability of THC indicates that the saturation of doubles bonds in the heptenoid chain gives more stability, but still the degradation is active due to the presence of phenolic groups and the active methylene group. The stability data of TMC indicates that the involvement of double bonds in the degradation of curcumin in cell medium. The introduction of the more sterically hindered cyclohexyl group at the C4 of the heptenoid chain favors less degradation and induces more stability to the curcuminoid and hence may be a potent agent for treatment of the cancer cells.

3.5. A pilot pharmacokinetic study of curcumin and TMC after a cassette i.p. dose of 10 mg/kg in mice

With this method, a pilot pharmacokinetic study was carried out in mice by a cassette i.p. dose of 10 mg/kg curcumin, DMCHC, and TMC. THC was not included in the pilot study because THC is a metabolite of curcumin. As shown in Fig. 4, a significant concentration of curcumin, DMCHC, TMC can be detected in 5 min after their i.p. administration and reach a C_{max} at 20 min and are detectable in plasma up to 4 h. Of note, as an *in vivo* metabolite of curcumin, THC was also detected at very low level in these first 20-min plasma samples (data not shown). This result suggested that all curcuminoids can be absorbed very quickly. The elimination half lives of curcumin, DMCHC and TMC are 0.4, 1.08 and 0.98 h, respectively. This result suggested that the elimination of DMCHC, TMC is slower than that of curcumin, which confirm our strategy to block two metabolic sites (C4 and phenolic group) to improve its pharmacokinetic properties without affecting the absorption

Table 3
The pharmacokinetic parameters of curcumin, DMCHC, TMC in mouse after a cassette intraperitoneal administration of 10 mg/kg of respective curcumin, DMCHC and TMC.

Parameter	Units	Curcumin	DMCHC	TMC
Lambda_z	1/h	1.686	1.686	1.686
HL_Lambda_z	h	0.411	1.082	0.975
T_{max}	h	0.333	0.333	0.333
C_{max}	μM	0.701	1.736	0.785
C_{last}	μM	0.001	0.046	0.025
AUC_{last}	h μM	0.441	0.977	0.643
AUC_{all}	h μM	0.441	0.977	0.643
AUC_{INF_obs}	h μM	0.441	1.049	0.678
Vz_{-F_obs}	L/kg	36.509	32.061	48.937
Cl_{-F_obs}	L/h/kg	0.017	0.006	0.010

properties. Consistent with their prolonged elimination half life, and increased the area under curve (AUC) of TMC (0.643 μM h, 1.5-fold), and of DMCHC (0.977 μM h, 2.3-fold) compared to that of curcumin (0.444 μM h), the C_{max} of DMCHC is higher (1.736 μM) than that of curcumin (0.701 μM) despite there is no difference in the C_{max} between curcumin and TMC. All relative PK parameters of curcumin, DMCHC and TMC retrieved from the non-compartment using Winnolin are listed in Table 3.

4. Conclusion

A sensitive LC–MS/MS method for simultaneous quantification of curcumin, THC, TMC and DMCHC with an LLOQ of 5.0 ng/mL for curcumin and 1.0 ng/mL for other three curcuminoid was developed in cell medium and mouse plasma, respectively. The stability study in these matrices at 37 °C using this method demonstrated an increase in stability in the order of curcumin, THC, TMC and DMCHC, which is consistent with their structural optimization to block the three metabolic sites of curcumin: conversion of α,β-unsaturated ketone to saturated ketone (THC), replacement of phenolic group with methoxy group, and substitution of hydrogen of methylene with methyl group (TMC) or relative steric group, e.g. cyclohexyl (DMCHC). Notably, a pilot pharmacokinetics of a cassette i.p. dosing of curcumin, DMCHC, TMC using this method demonstrated prolonged elimination half lives, increased AUC and higher C_{max} of TMC and DMCHC in mice compared to those of curcumin.

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