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Development of a Validated UPLC-TQD-MS Method for Bioavailability Study of Curcuminoids and the Metabolite in Rat Plasma

			
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ABSTRACT

A specific and rapid ultraperformance liquid chromatography (UPLC)–MS/MS method has been developed and validated for the estimation of curcuminoids in rat plasma, using biochanin as internal standard (IS). Also standardized the quantification methods of major metabolites of curcuminoids such as Curcumin-o-glucuronide, Hexahydrocurcumin, Tetrahydrocurcumin, and curcumin sulfate. The assay procedure involved SPE extraction of a curcuminoid and IS from rat plasma. The recovery of curcumin, demethoxycurcumin, bisdemethoxycurcumin and IS in rat plasma were 92.68%, 94.98%, 90.31% and 97.06%, respectively. The resolution of peaks was achieved with water containing 0.1% Formic acid/acetonitrile (55:45, v/v) on an Ambient BEH C₁₈ (2.1 x 50 mm), 1.7μ column.



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INTRODUCTION

“The spice herb, turmeric (*Curcuma longa* L.) contains curcuminoids, namely, curcumin, demethoxycurcumin and bisdemethoxycurcumin, essential oils and a biologically active protein, turmerin. Curcuminoids are considered as the most biologically active constituents of turmeric and it comprises 2-8% of the dried rhizome¹⁻². Interest in these dietary polyphenols, especially curcumin, has grown in recent years due to its vast array of beneficial pharmacological effects including antioxidant, anti-inflammatory, anticarcinogenic, hypocholesterolemic, antibacterial, wound healing, antispasmodic, anticoagulant, antitumor, antiangiogenic and hepatoprotective activities³. The pleiotropic nature of turmeric (and, by extrapolation, of curcumin) was known to Ayurvedic practitioners of ancient India⁴. There are considerable public and scientific interest in the use of diet-derived phytochemicals to combat or prevent human diseases and curcumin is currently a leading agent. Curcumin has also been reported a more potent free radical scavenger than vitamin E⁵. For these reasons, turmeric has been widely used as a food additive, condiment, and health food and even in cosmetics. Furthermore, data obtained in multiple preclinical models, as well as in preliminary clinical trials, have documented minimal toxicity of curcumin, even at relatively high doses. However, the clinical advancement of this promising molecule has been hindered by its poor water solubility, short biological half-life, and low bioavailability after oral administration². Efficient first-pass metabolism and some degree of intestinal metabolism, particularly glucuronidation and sulfation of curcumin, might explain its poor oral systemic availability^{2,6}. The absorption, metabolism, and tissue distribution of curcumin after oral administration of 400, 80 and 10 mg/kg body weight of curcuminoids in rats have been studied⁷⁻¹⁰. Owing to its wide spectrum nutritional as well as therapeutic effects, a number of preclinical and clinical multi-disciplinary evaluation works is currently underway to establish its clinical utility. Various HPLC methods are available in the literature for determination of curcuminoids¹¹⁻²¹. HPLC-MS methods also reported providing quantitation of curcuminoids²²⁻²⁴. This communication describes a rapid, specific, robust, and validated LC-MS/MS method for the quantification of curcuminoids in rat plasma. The speed of sample preparation and analysis, selectivity and sensitivity proved to be satisfactory.

MATERIALS AND METHODS

Chemicals and reagents

The USP standard of curcuminoids, Cat No1151866 (300 mg) were purchased from Sigma-Aldrich Ltd. The purity of standard was 77.00%, 18.55% and 3.50% for curcumin (C), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) respectively. Curcumin-*o*-glucuronide, Hexahydrocurcumin, Tetrahydrocurcumin and biochanin (IS) were purchased from Sigma-Aldrich Ltd (St Louis, USA). Curcumin sulfate was purchased from TLC Pharmachem., Inc., (Canada). Acetonitrile (LC-MS grade), methanol and formic acid were purchased from E Merck Limited (Mumbai, India).

Instrumentation and chromatographic conditions

An LC system (Waters, Corporation, Milford, U.S.A) consisting of an Acquity High-Performance LC and electrospray chemical ionization tandem mass spectrometer (ESCI-MS/MS; Waters) was used. ESCI-MS/MS was performed in the positive mode for curcuminoids and negative mode for curcumin metabolites: capillary voltage was 3Kv and cone voltages were 25V and 38V, respectively; drying gas (N₂) flow rate was 500 l/h; the ionization sources were 150⁰C; and the desolvation temperature was 350⁰C.

Multiple reaction monitoring, using the precursor→product combination of *m/z* 369.22→177.06, *m/z* 339.21→147.09, *m/z* 309.2→147.07 *m/z* 543.25→113.06 *m/z* 373.205→179.079, *m/z* 371.125→98.951, *m/z* 493.143→195.01 and 283.10→268.06 was used to quantify curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcumin glucuronide, hexahydrocurcumin, tetrahydrocurcumin, curcumin sulfate and the biochanin respectively. The samples were separated on Ambient BEH C₁₈ (2.1 x 50 mm), 1.7μ column. The system was run in an isocratic mode with a mobile phase consisting of acetonitrile/ water containing 0.1% Formic acid in the ratio (45:55, v/v)²⁰. The flow rate was 0.2 ml/min. Sample detection was achieved at 420 nm, and injection volumes were 5μl. Data acquisition and quantitation were performed using Mass Lynx software version 4.1.

The mobile phase was duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min prior to use. Separations were performed at room temperature.

Auto-sampler carry-over was determined by injecting to the highest calibration standard followed by a blank. No carry-over was observed, as indicated by the absence of curcuminoid and biochanin peaks in the blank.

Preparation of standard and internal standard solutions

Stock solutions of curcuminoid and IS were prepared in methanol (HPLC grade) at a concentration of 100 µg/ml separately. The curcuminoid stock solution was diluted with methanol to obtain working solutions of concentration ranging from 0.2 to 20 µg/ml. IS working solution (4µg/ml) was also prepared by diluting the stock solution with methanol. All the prepared solutions were protected from light using an amber-colored volumetric flask. Standard solutions (2, 5, 10, 50, 100, 200, 400 and 800 ng/ml) were prepared by spiking 50 µl of each working solution (0.04, 0.1, 0.2, 1, 2, 4, 8 and 16 µg/ml) into 900µl of blank plasma and 50µl of IS. For each validation and assay run, the calibration standards were freshly prepared from the working solutions. Quality control (QC) samples at three different levels were independently prepared at concentrations of 10 ng/ml (LOQ QC), 100 ng/ml MQC (medium QC) and 800 ng/ml of HQC (high QC) curcuminoid in the same way. IS concentration was kept constant at 200 ng/ml throughout the study.

Availability of Plasma

Blank, drug-free plasma samples were collected from an adult, healthy male Sprague-Dawley rats at animal house of Arjuna Natural Extracts Ltd. Plasma was harvested by centrifuging the blood at 2000 Xg for 5 min and stored frozen at -80±10°C until analysis.

Sample preparation

Fifty microliters of standard, unknown and QC samples were pipetted using micropipette into 2 ml Eppendorf microcentrifuge tubes. To each tube, 900 µl of plasma was added. The tubes were capped and vortexed for the 30s. Fifty microliters of IS were added in each tube. The tubes were capped and mixed as described above. The sample was loaded on a SPE cartridge previously activated with 1.5 ml of 0.05 % acetic acid in methanol and washed with 1.5 ml of 0.05 % acetic acid in water. After sample loading, the SPE tubes were washed with 1.5 ml of 0.05 % acetic acid in water and the analytes were eluted with 3 x 1 ml of methanol: acetonitrile (1:1) in a test

tube. The organic phase was evaporated in a high performance personal evaporating system (Genevac UK). The residue was reconstituted with 1ml of acetonitrile:water containing 0.1% Formic acid (1:1) and transferred into a micro-vial. A 5 μ l aliquot was injected into the chromatographic system.

Recovery

The recovery of a curcuminoid and IS, through SPE cartridge extraction procedure, was determined by comparing the peak areas of the analytes extracted from replicate QC samples (n = 6) with the peak areas of analytes from post-extracted plasma blank samples spiked at equivalent concentrations⁷⁻⁹. Recoveries of curcuminoid were determined at concentration 100 ng/ml, whereas the recovery of the IS was determined at a single concentration of 200 ng/ml.

Validation Procedures

The method was validated for selectivity, precision, accuracy, linearity, LLOQ and extraction efficiency, according to the International Conference on Harmonization guidelines.

Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for the analyte and IS using the proposed extraction procedure and chromatographic-MS conditions.

Matrix effect

The effect of rat plasma constituents over the ionization of curcuminoid and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n=6) with the response of analytes from neat standard samples (50 μ l of required working stock sample spiked into 900 μ l of methanol instead of blank plasma) at equivalent concentrations²⁵⁻²⁷. Peak area (A) of the analyte in spiked blank plasma with a known concentration (MQC) was compared with the corresponding peak area (B) obtained by direct injection of standard in the mobile phase. The ratio (A/B $\times$ 100) is defined as the matrix effect. The matrix effect for curcuminoid was determined at QC low, QC medium and QC high concentrations, viz., 10, 100 and 800 ng/ml whereas the matrix effect over the IS was determined at a single concentration of 200 ng/ml.

Typical MRM chromatogram of Curcuminoid and IS shown in fig.2.

Calibration curve

The calibration curve was acquired by plotting the ratio of peak area of curcuminoid to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 2, 5, 10, 50, 100, 200, 400 and 800 ng/ml. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curves of bisdemethoxycurcumin, demethoxycurcumin, curcumin and curcumin metabolites were linear over the concentration range of 2–800 ng/ml (r^2 , 0.994), 2–800 ng/ml (r^2 , 0.996), 2-800 ng/ml (r^2 , 0.997), 1-1000 ng/ml (r^2 , 0.997 for curcumin-*o*-glucuronide), 50-1000 ng/ml (r^2 , 0.998 for Hexahydrocurcumin) and 50-1000 ng/ml (r^2 , 0.999 for Tetrahydrocurcumin) respectively. The acceptance criteria for each back-calculated standard concentration were $\pm 15\%$ deviation from the nominal value except at LLOQ, which was set at $\pm 20\%$ (ICH). Calibration curve of curcuminoid, metabolites, and biochanin are shown in fig.3.

Precision and accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates at three different QC levels, i.e., 10, 100 and 800 ng/ml. The inter-day assay precision was determined by analyzing the three levels QC samples on six different runs. The criteria for acceptability of the data included accuracy within $\pm 15\%$ standard deviation (S.D.) from the nominal values and a precision of within $\pm 15\%$ relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed $\pm 20\%$ of accuracy as well as precision (ICH).

RESULTS AND DISCUSSION

The LC-MS/MS procedure was optimized with a view to developing a bioanalytical method to quantify curcuminoids & metabolites in the presence of IS (Biochanin) from rat plasma. Biochanin is commercially available in high purity, and it is stable and nonreactive with the sample or mobile phase. Three curcuminoids and four metabolites we used as a standard in the present study showed separate peaks in the total ion chromatogram (TIC) at 1.99, 2.24, 2.53, 1.03, 1.3, 1.22 and 0.95 min for bisdemethoxycurcumin, demethoxycurcumin, Curcumin, hexahydrocurcumin, tetrahydrocurcumin, curcumin sulfate and curcumin-*o*-glucuronide

respectively. The bisdemethoxycurcumin, demethoxycurcumin and curcumin and metabolites were also appeared in the total ion chromatogram TIC. Fig.1 &2 - Typical UPLC-TQD-MS/MS chromatograms showing curcuminoids, metabolites, blank plasma & IS.

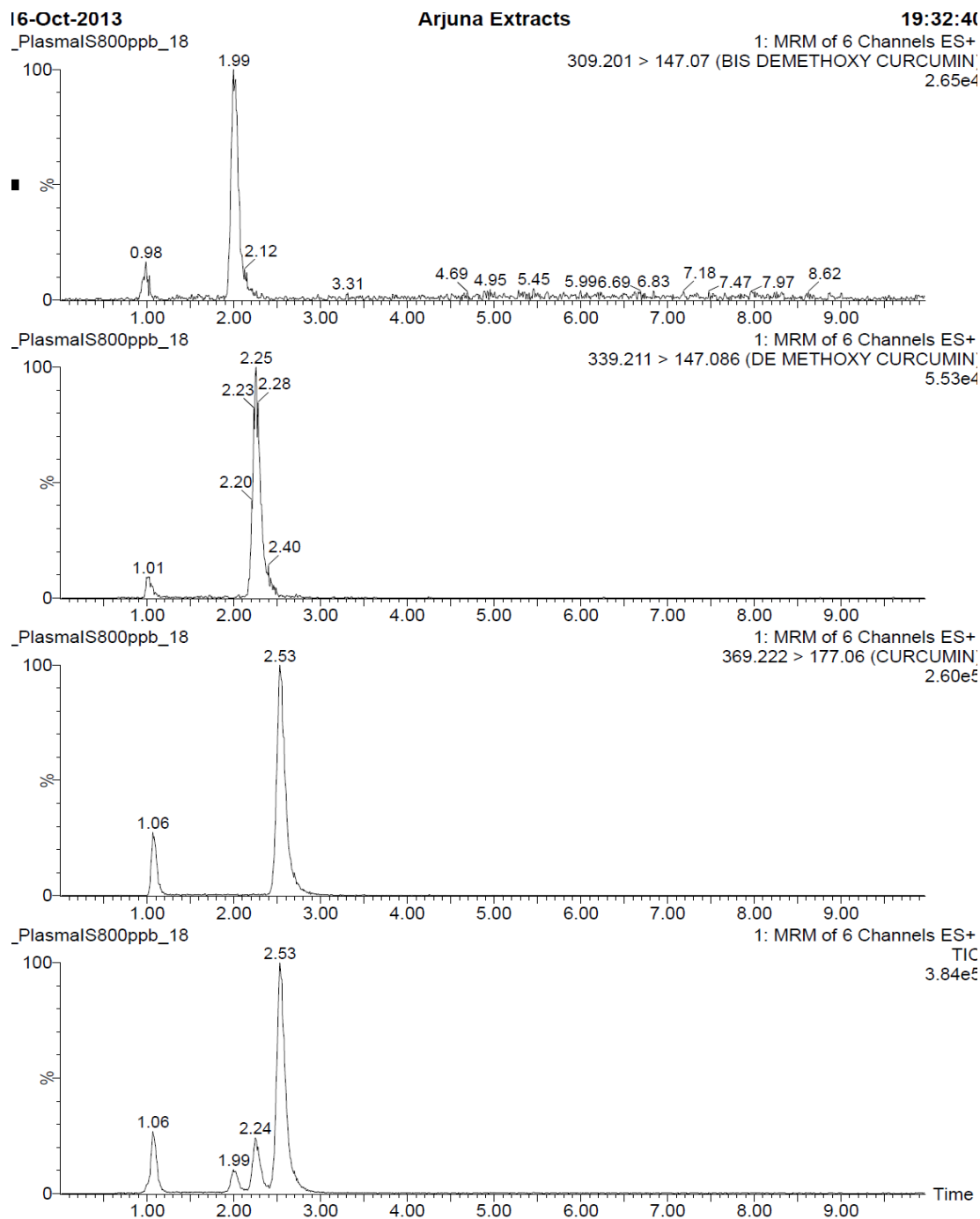


Figure 1 (a): MRM chromatogram of bisdemethoxycurcumin, demethoxycurcumin, curcumin & Total ion chromatogram (TIC) of Curcuminoids

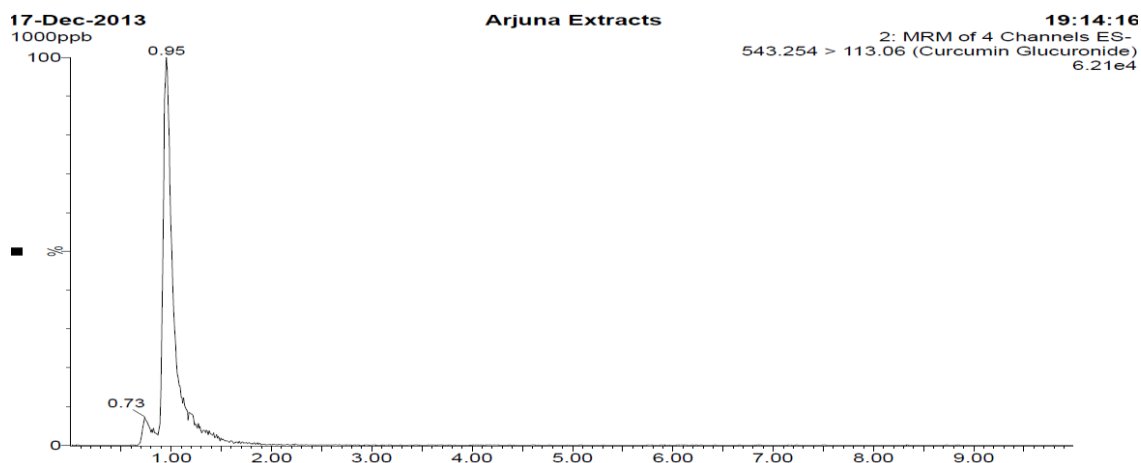


Figure1 (b): MRM chromatogram of Curcumin-o-glucuronide

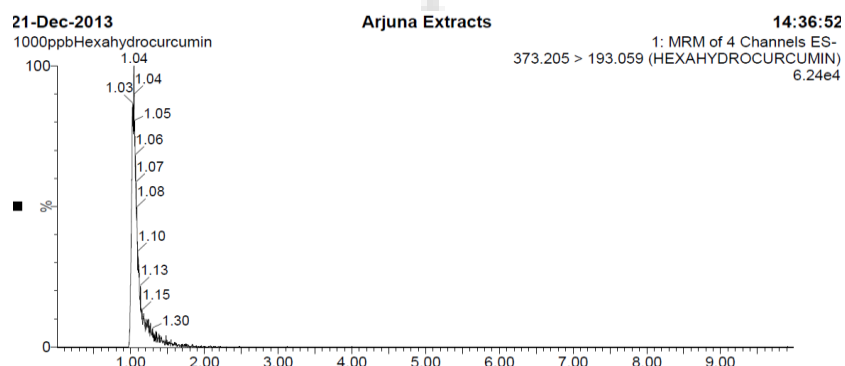


Figure1 (c): MRM chromatogram of Hexahydrocurcumin.

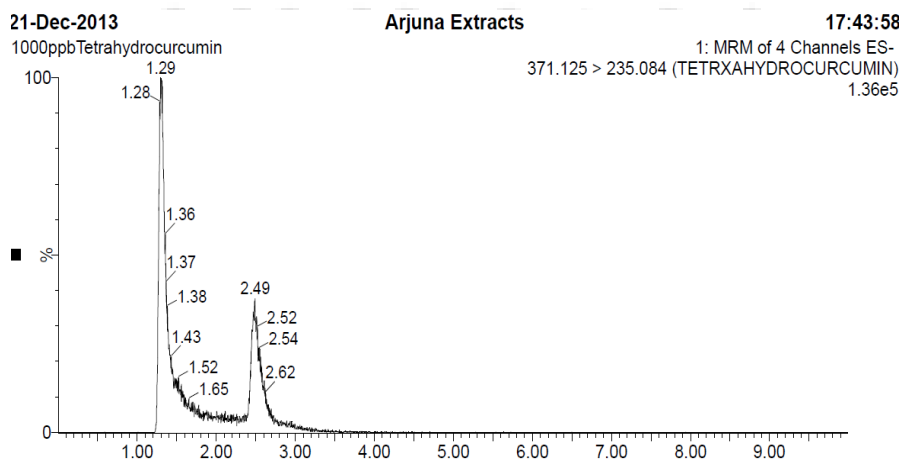


Figure1 (d): MRM chromatogram of Tetrahydrocurcumin.

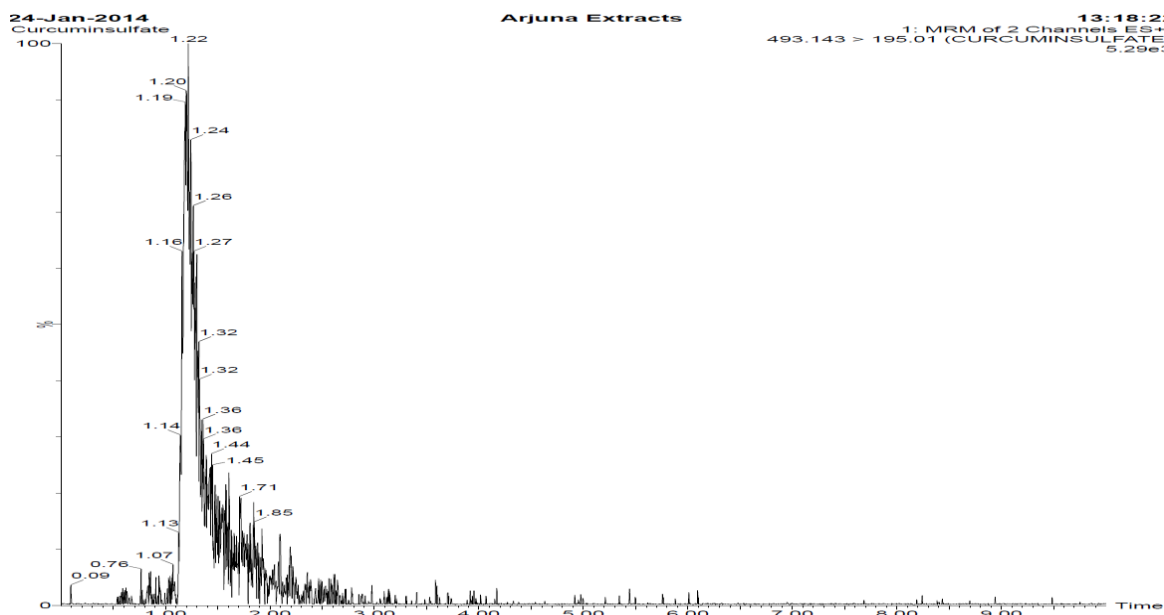


Figure1(e). MRM chromatogram of curcumin sulfate

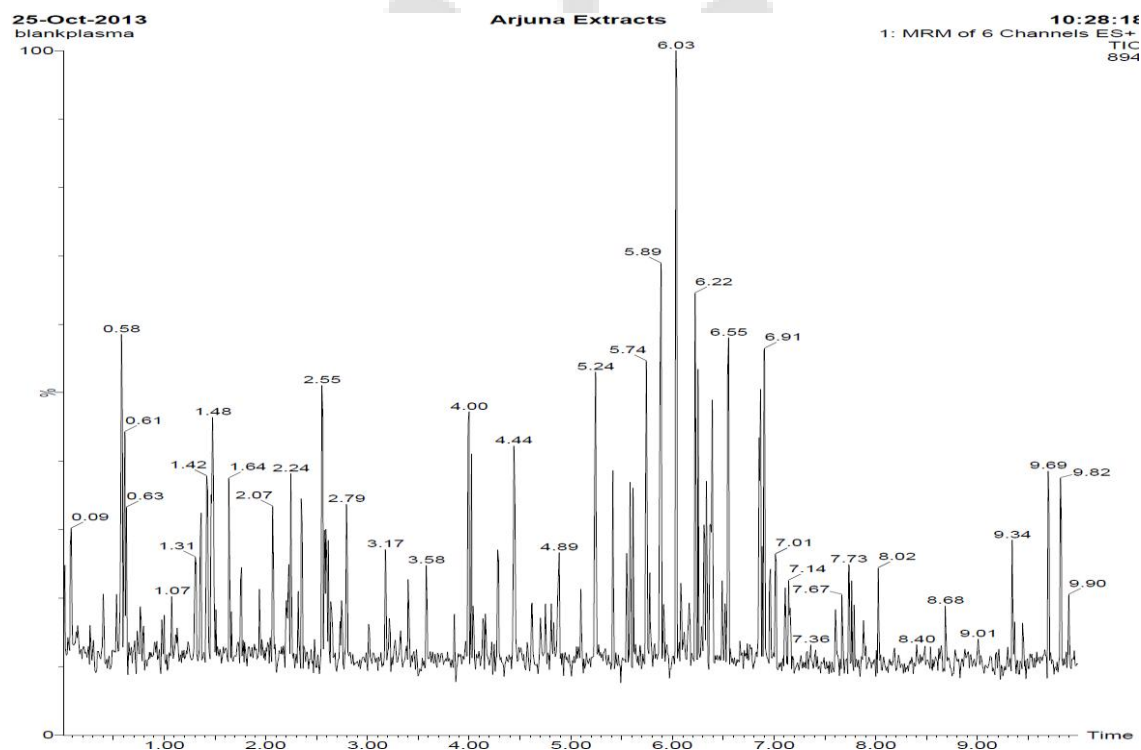


Figure 2 (a): MRM chromatograms of Blank plasma

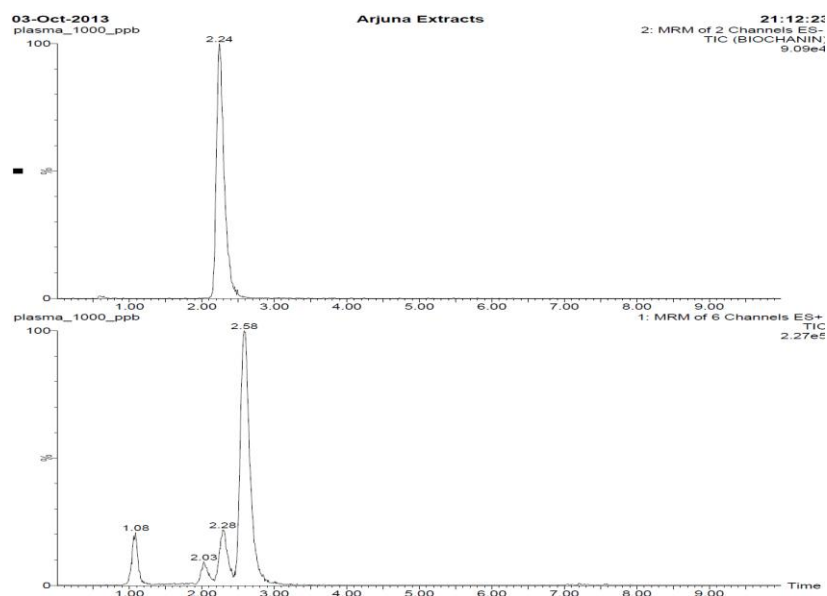


Figure 2 (b): MRM chromatograms of Biochanin (IS) & Curcuminoids.

A method for the determination of curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumin metabolites by ultra-performance liquid chromatography (UPLC)-MS/MS has not been reported, prior to this investigation, in which curcuminoids and metabolites have been quantified on the basis of their major fragment. The quantification of the analytes was achieved by using MRM which makes the proposed method most acceptable.

Liquid chromatography

Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate and formic acid with variable pH range of 4.5–6.5, along with altered flow-rates (in the range of 0.2–0.4 ml/min) were tested for complete chromatographic resolution of curcuminoid and IS (data not shown). Mobile phase comprising of water containing 0.1% Formic acid: Acetonitrile (55:45, v/v) was delivered at a flow rate of 0.2 ml/min was found to be suitable during LC optimization and enabled the determination of electrospray response for curcuminoid and IS. Experiments were also performed with different C₁₈ columns and found that chromatographic resolution, selectivity and sensitivity were good with Ambient BEH C₁₈ (2.1 x 50 mm), 1.7μ column.

Mass spectrometry

In order to optimize ESI and APCI conditions for curcuminoids, metabolites and IS quadrupole full scans were carried out in positive ion detection mode in curcuminoids and negative ion detection mode in metabolites and IS. During a direct infusion experiment, the mass spectra for curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcumin-*o*-glucuronide, hexahydrocurcumin, tetrahydrocurcumin, curcumin sulphate and IS revealed peaks at m/z 369, 339, 309, 543, 373, 371 and 283, respectively as protonated molecular ions $[M-H]^+$ and deprotonated molecular ions $[M-H]^-$.

Recovery

The results of the comparison of pre-extracted standard versus post-extracted plasma standard were estimated for curcumin, demethoxycurcumin and bisdemethoxycurcumin at 100 ng/ml and the absolute mean recovery were 92.68 %, 94.98%, and 90.31% respectively. The absolute recovery of IS at 200 ng/ml was 97.01%.

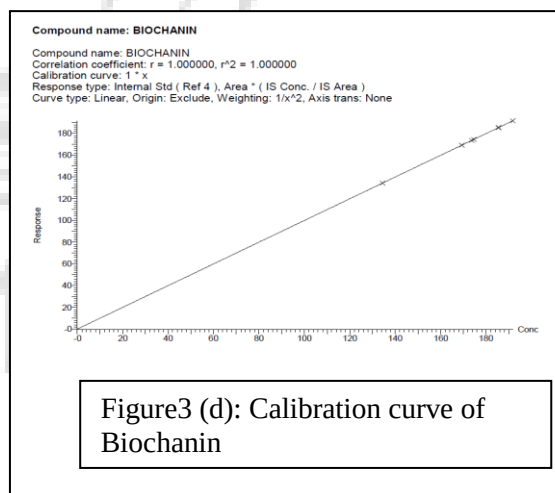
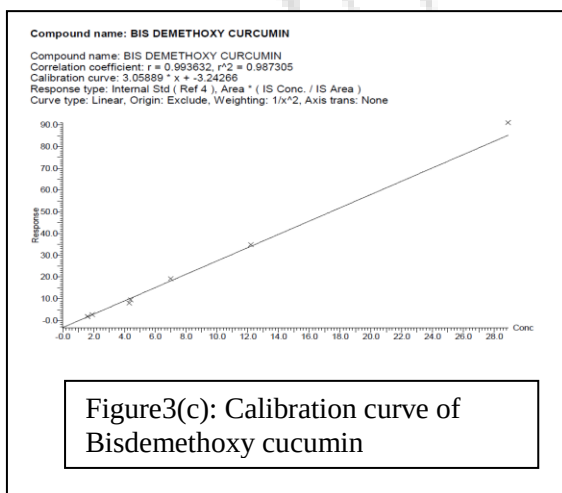
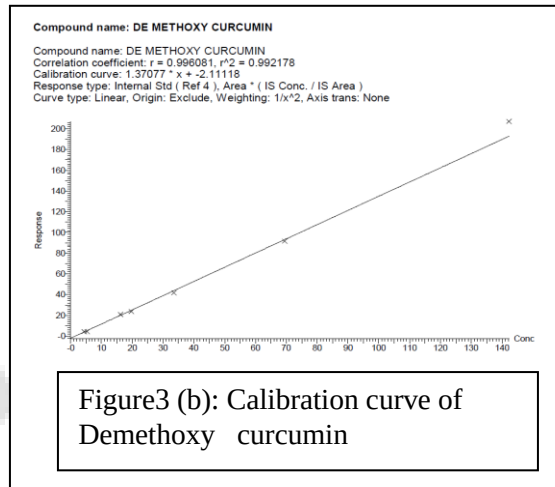
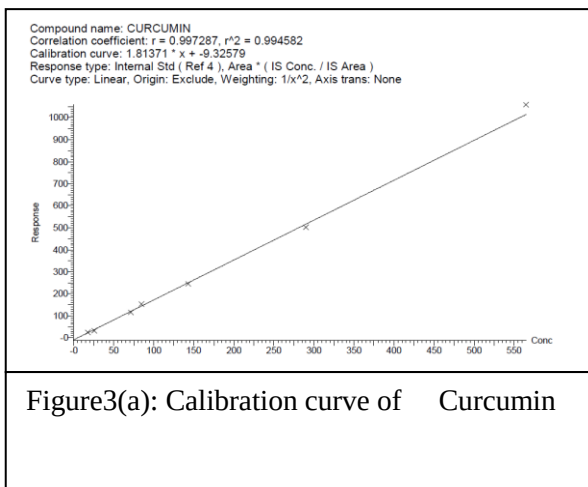
Matrix effect, specificity, and selectivity

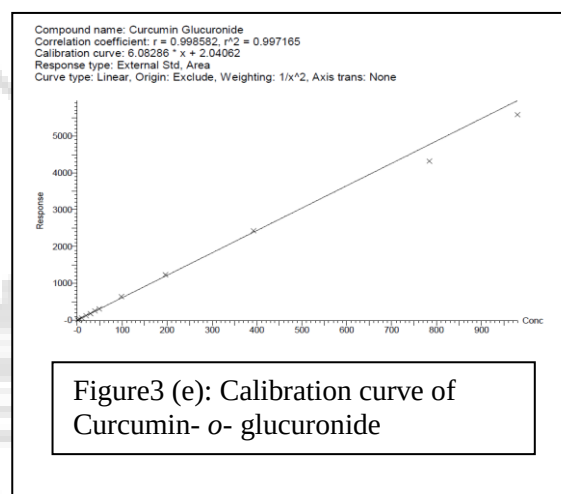
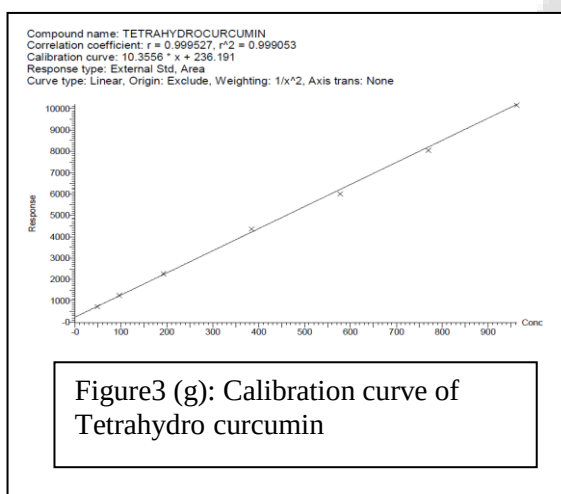
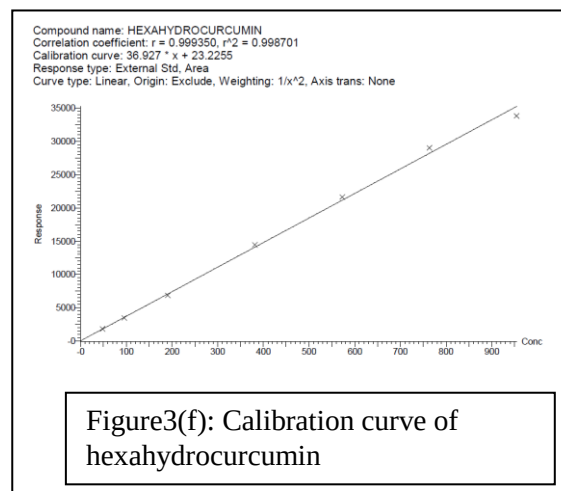
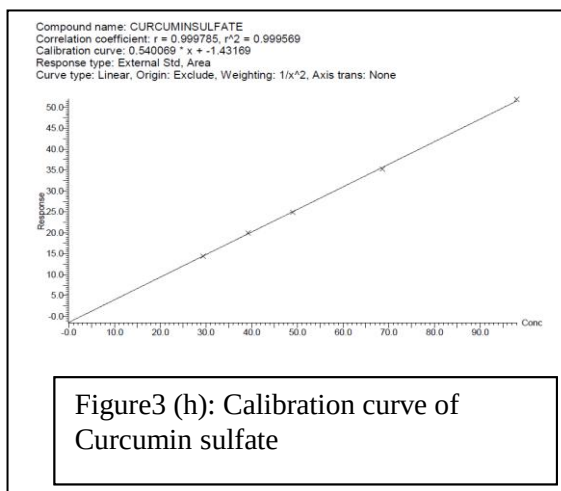
In this study, the matrix effect was evaluated by analyzing QC medium (100 ng/ml). The matrix effect ($A/B \times 100$) for curcumin was 96.78% (% RSD: 3.14; $n = 5$), and for DMC it was 94.31% (% RSD: 4.05; $n=5$) and for BDMC it was 92.13% (% RSD: 3.89; $n=5$) Percent RSD < 5 suggested that the method was free from matrix effect. In the present study, the specificity and selectivity have been studied by using independent plasma samples from six different rats. No significant interference at the retention time of the drug or IS was found. The retention time of curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcumin-*o*-glucuronide, hexahydrocurcumin, tetrahydrocurcumin and IS were at 2.53, 2.24, 1.99, 0.95, 1.03, 1.3 and 2.24 min respectively. The total chromatographic run time was 10 min.

Calibration curve

The plasma calibration curve was constructed using eight calibration standards (viz., 2–800 ng/ml). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining

the best fit of peak-area ratios (peak area analyte / peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The lowest concentration with R.S.D. < 20% was taken as LLOQ and was found to be 2ng/ml. Fig.3 - showing the calibration curves of curcuminoids, metabolites, & IS.





Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 1 and Table 2. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Table 1

Intra-day assay precision and accuracy for curcuminoid in rat plasma ($n = 6$). The intra-day assay precision and accuracy were estimated by analyzing six replicates at three different concentrations, i.e., 10, 100 & 800 ng/ml.

Nominal Concentration	Day	Observed Concentration curcumin (ng/mL,	Precision * (%) curcumin	Accuracy** (%)curcumin	Observed Concentration DMC (ng/mL, mean± S.D.)	Precision * (%) DMC	Accuracy ** (%)DMC	ObservedConcentration B DMC (ng/mL,mean ± S.D	Precision * (%)B BDMC	Accuracy** (%) BDMC
10	Day 1	7.45±0.71	9.53	96.75	1.6±0.2	12.5	86.49	0.32±0.04	12.5	91.43
	Day 2	7.22±0.65	9	93.77	1.62±0.15	9.26	87.56	0.38±0.05	13.16	108.5
	Day 3	7.57±0.61	8.06	98.31	1.75±0.18	10.29	94.59	0.36±0.04	11.11	102.86
100	Day 1	80.33±5.7	7.1	104.32	19.32±1.0	5.16	104.59	3.8±0.6	12.8	108.57
	Day 2	81.82±5.6	6.84	106.26	20.65±2.6	12.59	111.62	3.42±0.6	13.69	97.71
	Day 3	78.7±5.7	7.24	102.21	19.1±2.3	12.04	103.24	3.15±0.4	9.96	90
800	Day 1	689.75±19.9	2.89	111.97	158.58±12	7.57	107.15	29.5±2.3	7.79	105.36
	Day 2	630.4±10.9	1.72	102.34	156.73±3.23	2.06	105.89	30.6±2.5	8.17	109.29
	Day 3	631.75±9.2	1.46	102.56	155.87±4.5	2.89	105.32	30.35±3.5	11.53	108.39

* Expressed as % R.S.D. (S.D./mean) × 100.

** Calculated as (mean determined concentration/nominal concentration) × 100.

Table 2: Inter-day assay precision and accuracy for curcuminoid in rat plasma

Nominal concentration (ng/mL)	Observed Concentration curcumin (ng/mL, mean $\pm$ S.D.)	Precision *(%) curcumin	Accuracy ** (%) curcumin	Observed Concentration DMC (ng/mL, mean $\pm$ S.D.)	Precision *(%) DMC	Accuracy ** (%) DMC	Observed Concentration B DMC (ng/mL, mean $\pm$ S.D)	Precision *(%) B DMC	Accuracy ** (%) B DMC
10	7.41 $\pm$ 0.67	9.04	96.23	1.66 $\pm$ 0.19	11.45	89.73	0.35 $\pm$ 0.054	15.43	100
100	80.28 $\pm$ 5.86	7.36	104.26	19.69 $\pm$ 2.1	10.67	106.43	3.46 $\pm$ 0.5	14.45	98.86
800	650.63 $\pm$ 14.56	2.23	105.62	157.06 $\pm$ 8.56	5.45	106.5	30.15 $\pm$ 2.9	9.62	107.68

n = 3 days with six replicates per day.

* Expressed as % R.S.D. (S.D./mean) $\times$ 100.

** Calculated as (mean determined concentration/nominal concentration) $\times$ 100.

Application of the method

The rat plasma samples generated following oral administration of curcuminoids were analyzed by the newly developed and validated method along with QC samples. All the QCs met the acceptance criteria (data not shown). The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of curcuminoids in rats.

CONCLUSION

A simple, rapid and sensitive UPLC-MS/MS method was developed and validated for the determination of curcuminoids and metabolites in rat plasma. This method has shown a high degree of simplicity, accuracy, sensitivity, reproducibility and also provides short analysis time (10 min.). The sensitivity of the method allowed the pharmacokinetic study of curcuminoids and

metabolites in plasma after oral administration of curcuminoid sample in male Wistar rats. The method was validated in terms of specificity, accuracy, precision and sensitivity and utilized for the determination of curcuminoids and metabolites in either individually or simultaneously in plasma (rat). From the results of all the validation parameters and applicability of the assay, we can conclude that the present method can be useful for preclinical pharmacokinetic studies of curcuminoids and metabolites with desired precision and accuracy along with high throughput.

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