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Determination of Curcumin in Rat Plasma by Liquid–liquid Extraction using LC–MS/MS with Electrospray Ionization: Assay Development, Validation and Application to a Pharmacokinetic Study

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Abstract

A simple, specific and rapid LC–MS/MS method has been developed and validated for the estimation of curcumin in rat plasma, using biochanin as internal standard (IS). The assay procedure involved liquid–liquid extraction of curcumin and IS from rat plasma. The recovery of curcumin and IS in rat plasma were 87.62 and 88.25%, respectively. The resolution of peaks was achieved with 0.01 M ammonium acetate (pH 5.5):acetonitrile (10:90, v/v) on a Supelco Discovery C18 column. The total chromatographic run time was 4 min. Specificity and matrix effect on ionization was determined and found that method was specific and there was no significant matrix effect. The method was proved to be accurate and precise at linearity range of 10–2000 ng/mL with a correlation coefficient (r) of ≥0.995. The MS/MS ion transitions monitored were 367→217 for curcumin and 283→268 for IS. The intra- and inter-day assay precision ranged from 2.79 to 8.20% and 4.15 to 7.76%, respectively; and intra- and inter-day assay accuracy was between 92.83–107.83% and 93.92–104.26%, respectively. Practical utility of this LC–MS/MS method was demonstrated in a pilot pharmacokinetic study in male Sprague–Dawley rats following intravenous administration of curcumin.

Keywords: Curcumin; Rat plasma; Validation; LC–MS/ MS; Pharmacokinetics

Introduction

Curcuma spp. contain turmerin, essential oils, and curcuminoids, including curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] which is a phenolic substance derived from dietary spice herb Curcuma longa. It is regarded as the most biologically active constituent of the spice turmeric and it comprises 2-8% of most turmeric preparations (Bisht and Maitra, 2009; Sharma et al., 2007). Interests in this dietary polyphenol 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, anti-angiogenic and hepatoprotective activities (Aggarwal and Harikumar, 2009; Pari et al., 2008). With regard to considerable public and scientific interest in the use of phytochemicals derived from dietary components to combat or prevent human diseases, curcumin is currently a leading agent. For these reasons, turmeric has been widely used as a food additive, condiment, and health food. Further, 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 administration (Sharma et al., 2007). Efficient first-pass metabolism and some degree of intestinal metabolism, particularly glucuronidation and sulfation of curcumin, might explain its poor oral systemic availability (Anand et al., 2007; Sharma et al., 2007). The absorption, metabolism, and tissue distribution of curcumin after oral administration of 400, 80 and 10 mg of curcumin in rats has been studied (Ravindranath and Chandrasekhara, 1981). Due to its wide spectrum nutritional as well as therapeutic effects, a lot of preclinical multi-disciplinary evaluation work is going on with curcumin to advocate its clinical utility.

Development of validated assay procedure in preclinical animal species is essential at various stages of drug discovery and development. So far, several assay methodologies have been reported for determination of curcuminoids using gas chromatography, capillary electrophoresis(Lechtenberg et al., 2004), HPLC (Asakawa et al., 1981; Heath et al., 2005; Pak et al., 2003) and LC-MS/MS (Marczylo et al., 2009; Yang et al., 2007). However, there is an ever-increasing need to have a battery of improved, faster, reproducible and reliable bio-analytical methods which ultimately may lead to variety of choices before the analysts. Therefore, we developed and validated a rapid, specific and robust LC-ESI-MS/MS method for the quantification of curcumin in rat plasma. The speed of sample preparation and analysis, selectivity and sensitivity proved to be satisfactory. In addition, using the method described above, the pharmacokinetic profile of curcumin in rats has been determined following intravenous bolus administration.

Experimental

Chemicals and reagents

Curcumin and biochanin (IS) were purchased from Sigma Aldrich Ltd (St Louis, USA). Chemical structure of curcumin and biochanin is shown in Figure 1. HPLC grade acetonitrile, methanol, ethyl acetate and dichloromethane were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India). Dimethyl sulfoxide was purchased from Thomas baker chemicals Pvt. Limited (Mumbai,

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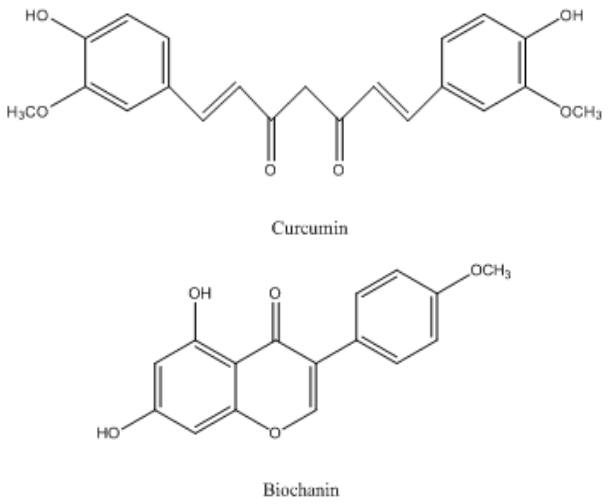

Figure 1: Structural representation of curcumin and biochanin.

India). Ammonium acetate and glacial acetic acid (GAA) AR were purchased from E Merck Limited (Mumbai, India). Ultra pure water was obtained from a Sartorious Arium 611 system. Heparin sodium injection I.P. (1000 IU/mL, Biologicals E. Limited, Hyderabad, India) was purchased from local pharmacy. Blank, drug free plasma samples were collected from adult, healthy male Sprague–Dawley rats at Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood (25 IU/mL) at 2000×g for 10 min. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals.

Instrumentation and chromatographic conditions

HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin- Elmer instruments, Norwalk, USA) was used to inject 10 µL aliquots of the processed samples on a Supelco Discovery C18 column (4.6 × 50 mm, 5.0 µm). The system was run in isocratic mode with mobile phase consisting of 0.01 M ammonium acetate (pH 5.5) and acetonitrile in the ratio of 10:90 (v/v) at a flow rate of 0.5 mL/min. 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 the highest calibration standard then a blank sample. No carry-over was observed, as indicated by the lack of curcumin and biochanin peaks in the blank sample.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at -5500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 40, 10, 20 and 8, respectively. Compounds parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were -60, -15, -10, -10 V and -92, -12, -10, -8 V for curcumin and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI negative ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of *m/z* 367 precursor ion $[M–H]^-$ to the *m/z* 217 product ion for curcumin and *m/z* 283 precursor ion $[M–H]^-$ to the *m/z* 268 product ion for IS. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

Preparation of stock and standard solutions

The primary stock solution of the curcumin was prepared by dissolving 5 mg in 0.2 mL of dimethyl sulfoxide and diluting with methanol to a final concentration of 1000 µg/mL. The IS primary stock solution of 1000 µg/mL was prepared in methanol. Appropriate dilutions were made in methanol for curcumin to produce working stock solution of 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 µg/mL and on the day of analysis this set of stocks was used to prepare standards for the calibration curve. Another set of working stock solutions of curcumin was made in methanol at 16, 8, 0.4 and 0.1 µg/mL for preparation of QC samples. Individually QC and CC working stock solutions of curcumin were spiked into blank plasma for QC and CC samples. A working stock solution of IS (1 µg/mL) was prepared in methanol from primary stock solution of 1000 µg/mL in methanol. Calibration standards and QC samples were prepared by spiking 90 µL of control pooled rat plasma with the appropriate working solution of curcumin (10 µL) and IS (10 µL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by individually spiking control rat plasma at four concentration levels [10 ng/mL (lower limit of quantitation, LLOQ), 40 ng/mL (QC low), 800 ng/mL (QC medium) and 1600 ng/mL (QC high)] and stored at –80 ± 10°C until analysis.

Recovery

The recovery of curcumin and IS, through liquid–liquid 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 concentrations (Dams et al., 2003; Singh et al., 2008; Wahajuddin et al., 2009). Recoveries of curcumin were determined at QC low, QC medium and QC high concentrations viz., 40, 800, and 1600 ng/mL, whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

Sample preparation

A simple liquid–liquid extraction method was followed for extraction of curcumin from rat plasma. To 100 µL of plasma in a tube, 10 µL of IS solution (biochanin at 1 µg/mL in methanol), was added and mixed for 15 sec on a cyclomixer (Spinix Tarsons, Kolkata, India). Then 2 mL of dichloromethane/ethyl acetate (1/1, v/v) was added and the mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000×g on Sigma 3-16K (Frankfurt, Germany). The organic layer (1.6 mL) was separated and evaporated to dryness under vaccum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 µL of the mobile phase and 10 µL was injected onto analytical column.

Validation procedures

A full validation according to the FDA guidelines was performed for the assay in rat plasma (US DHHS, FDA, CDER. 2001).

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 analyte and IS using the proposed extraction procedure and chromatographic-MS conditions.

Matrix effect: The effect of rat plasma constituents over the

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ionization of curcumin 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 (10 µL of required working stock sample spiked into 90 µL of methanol instead of blank plasma) at equivalent concentrations (Dams and others 2003; Singh and others 2008; Wahajuddin and others 2009)[13-15]. The matrix effect for curcumin was determined at QC low, QC medium and QC high concentrations, viz., 40, 800 and 1600 ng/mL whereas

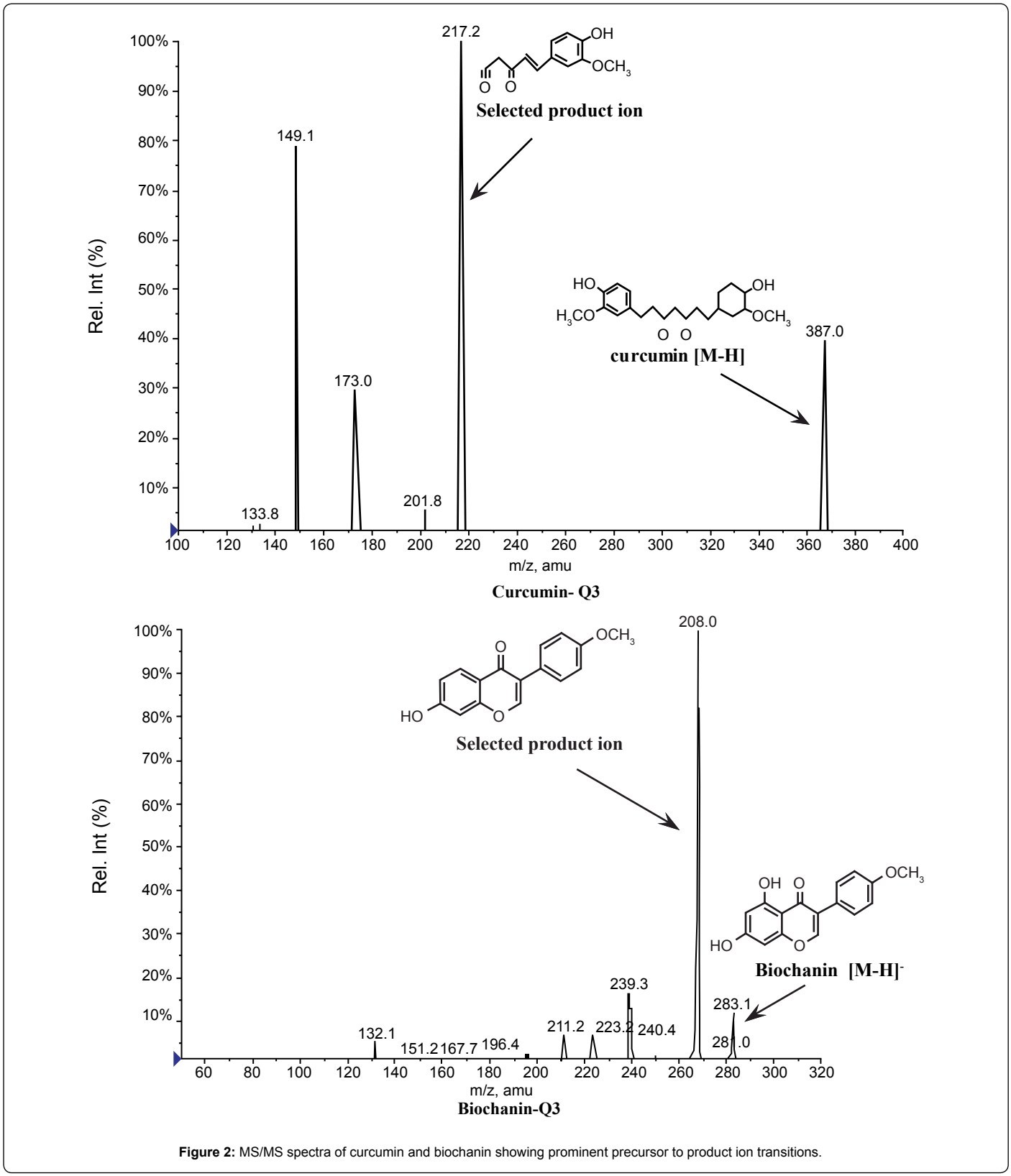

Figure 2: MS/MS spectra of curcumin and biochanin showing prominent precursor to product ion transitions.

the matrix effect over the IS was determined at a single concentration of 100 ng/mL.

Calibration curve: The calibration curve was acquired by plotting the ratio of peak area of curcumin to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 10, 20, 50, 100, 200, 500, 1000, 2000 ng/mL. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS, FDA, CDER. 2001).

Precision and accuracy: The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 10, 40, 800 and 1600 ng/mL. The inter-day assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% standard deviation (S.D.) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision (US DHHS, FDA, CDER. 2001).

Stability experiments: All stability studies were conducted at two concentration levels, i.e. QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 20 h to establish autosampler stability of analyte and IS. The peak areas of analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of curcumin in the biomatrix during 4 h exposure at room temperature in rat plasma (bench top) was determined at ambient temperature (25 ± 2°C). Freeze/thaw stability was evaluated up to three cycles. In each cycle samples were frozen for at least 12 h at –80 ± 10°C. Freezer stability of curcumin in rat plasma was assessed by analyzing the QC samples stored at –80 ± 10°C for at least 15 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., ±15% R.S.D.).

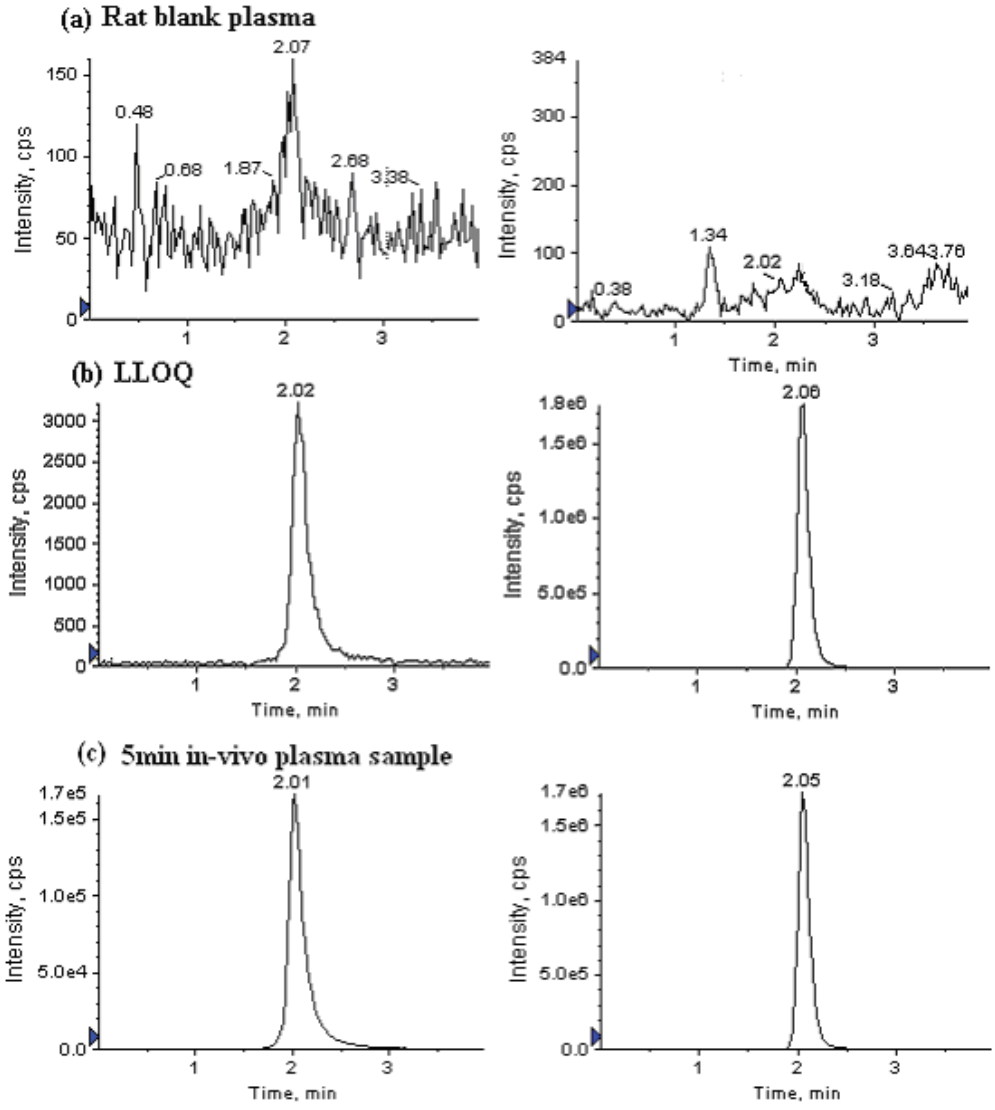

Figure 3: Typical MRM chromatograms of curcumin (left panel) and IS (right panel) in (a) rat blank plasma, (b) rat plasma spiked with curcumin at LLOQ (10 ng/mL) and IS (c) a 5 min *in vivo* plasma sample showing curcumin peak obtained following intravenous administration of curcumin.

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Application to a pharmacokinetic study in rats

A pharmacokinetic study was performed to show the applicability of newly developed and validated bioanalytical method. Study was performed in male Sprague–Dawley rats (n = 3, weight range 200–220 g). Curcumin was administered intravenously at a dose of 10 mg/kg. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 5, 15, 30, 45, 60, 120, 180 and 240 min post-dosing. Plasma was harvested by centrifuging the blood at 2000×g for 5 min and stored frozen at –80 ± 10°C until analysis. Plasma (100µL) samples were spiked with IS, and processed as described above. Along with the plasma samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among calibrators and unknown samples in the analytical run.

Results

Liquid chromatography

Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, acetic acid and formic acid with variable pH range of 4.5–6.5, along with altered flow-rates (in the range of 0.4–0.8 mL/min) were tested for complete chromatographic resolution of curcumin and IS (data not shown). Mobile phase comprising of 0.01 M ammonium acetate (pH 4.5):acetonitrile (10:90, v/v) was delivered at a flow rate of 0.5 mL/min was found to be suitable during LC optimization and enabled the determination of electrospray response for curcumin and IS. Experiments were also performed with different C18 columns and found that chromatographic resolution, selectivity and sensitivity were good with Supelco Discovery C18 column (4.6 × 50 mm, 5.0 µm).

Nominal Concentration (ng/mL)	Observed concentration (ng/mL, mean ± S.D.)	Precision [a] (%)	Accuracy [b] (%)
10	10.17 ± 0.21	2.05	101.67
20	19.27 ± 1.15	5.97	96.33
50	44.35 ± 1.06	2.39	88.70
100	107.67 ± 4.04	3.75	107.67
200	216.67 ± 1.53	0.71	108.33
500	489.33 ± 20.13	4.11	97.87
1000	925.33 ± 22.19	2.40	92.53
2000	1940 ± 70	3.61	97

[a] Expressed as % R.S.D. (S.D./mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 1: Precision and accuracy data of back-calculated concentrations of calibration samples for curcumin in rat plasma (n = 3).

Nominal concentration (ng/mL)	Observed concentration (ng/mL, mean ± S.D.)	Precision [a] (%)	Accuracy [b] (%)
10			
(Day-1)	10.58 ± 0.67	6.31	105.83
(Day-2)	9.98 ± 0.82	8.20	99.75
(Day-3)	10.72 ± 0.69	6.40	107.18
40			
(Day-1)	39.05 ± 2.93	7.50	97.63
(Day-2)	39.12 ± 3.20	8.17	97.79
(Day-3)	43.13 ± 1.24	2.88	107.83
800			
(Day-1)	742.67 ± 34.20	4.60	92.83
(Day-2)	761 ± 38.43	5.05	95.13
(Day-3)	750.50 ± 29.34	3.91	93.81
1600			
(Day-1)	1542 ± 43.09	2.79	96.77
(Day-2)	1614 ± 52.92	3.28	100.88
(Day-3)	1615 ± 82.64	5.12	100.94

[a] Expressed as % R.S.D. (S.D./mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 2: Intra-day assay precision and accuracy for curcumin in rat plasma (n = 6).

Mass spectrometry

In order to optimize ESI conditions for curcumin and IS, quadrupole full scans were carried out in negative ion detection mode. During a direct infusion experiment, the mass spectra for curcumin and IS revealed peaks at *m/z* 367 and 283, respectively as deprotonated molecular ions $[M–H]^-$. The product ion mass spectrum for curcumin shows the formation of characteristic product ions at *m/z* 149.2, 172.9 and 217.1 (Figure 2). Following detailed optimization of mass spectrometry conditions (provided in instrumentation and chromatographic conditions section), the *m/z* 367 precursor ion to the *m/z* 217 was used for quantification for curcumin. Similarly, for IS *m/z* 283 precursor ion to the *m/z* 268 was used for quantification purpose.

Recovery

The results of the comparison of pre extracted standards versus post-extracted plasma standards were estimated for curcumin at 40, 800 and 1600 ng/mL and the absolute mean recovery was 87.62%. The absolute recovery of IS at 100 ng/mL was 88.25%.

Validation procedures

Matrix effect, specificity and selectivity: In this study, the matrix effect was evaluated by analyzing QC low (40 ng/mL), QC medium (800 ng/mL) and QC high samples (1600 ng/mL). Average matrix effect values obtained were 2.53, 3.24 and 2.88% at QC low, QC medium and QC high, respectively. Matrix effect on IS was found to be 0.33% at tested concentration of 100 ng/mL.

In the present study, the specificity and selectivity has been studied by using independent plasma samples from six different rats.

Figure 3 shows a typical overlaid chromatogram for the control rat plasma (free of analyte and IS), rat plasma spiked with curcumin at LLOQ and IS and an in vivo rat plasma sample obtained at 5 min after intravenous administration of curcumin. No significant interference at the retention time of the drug or IS was found. The retention time of curcumin and IS were 2.02 and 2.05 min, respectively. The total chromatographic run time was 4 min.

Calibration curve: The plasma calibration curve was constructed using eight calibration standards (viz., 10–2000 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 average regression (n = 3) was found to be ≥ 0.997. The lowest concentration with R.S.D. < 20% was taken as LLOQ and was found to be 10 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 88.70–108.33; while the % precision values ranged from 0.71–5.97 (Table 1).

Accuracy and precision: Accuracy and precision data for intra- and inter-day plasma samples are presented in Table 2 and Table 3. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Stability: The predicted concentrations for curcumin at 40 and 1600 ng/mL samples deviated within the nominal concentrations in a battery of stability tests, viz., in-injector (20 h), bench-top (4 h), repeated three freeze/thaw cycles and at –80 ± 10°C for at least for 15 days (Table 4). The results were found to be within the assay variability limits during the entire process.

Nominal concentration (ng/mL)	Observed concentration [a] (ng/mL, mean ± S.D.)	Precision [b] (%)	Accuracy [c] (%)
10	10.43 ± 0.76	7.28	104.26
40	40.43 ± 3.14	7.76	101.08
800	751.39 ± 33.04	4.40	93.92
1600	1591.11 ± 65.97	4.15	99.44

[a] n = 3 days with six replicates per day.
[b] Expressed as % R.S.D. (S.D./mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 3: Inter-day assay precision and accuracy for curcumin in rat plasma.

Nominal concentration (ng/mL)	Stability	Mean ± S.D.[a] n = 6 (ng/mL)	Precision [b] (%)	Accuracy [c] (%)
40	0 h (for all)	39.05 ± 2.93	7.50	97.63
	20 h (Auto-sampler)	37.33 ± 1.45	3.89	95.60
	4 h (bench top)	42.12 ± 1.56	3.71	107.85
	3rd freeze-thaw	42.07 ± 2.25	5.34	107.73
	15 day at -80ºC	42.27 ± 1.87	4.42	108.24
1600	0 h (for all)	1548.33 ± 43.09	2.78	96.77
	20 h (Auto-sampler)	1620 ± 71.27	4.40	104.63
	4 h (bench top)	1546 ± 75.37	4.87	99.85
	3rd freeze-thaw	1544 ± 83.55	5.41	99.72
	15 day at -80ºC	1551.67 ± 64.32	4.15	100.22

a Back calculated plasma concentrations.
b Expressed as % R.S.D. (S.D./mean) × 100.
c Calculated as (mean determined concentration/nominal concentration) × 100.

Table 4: Stability of curcumin in rat plasma.

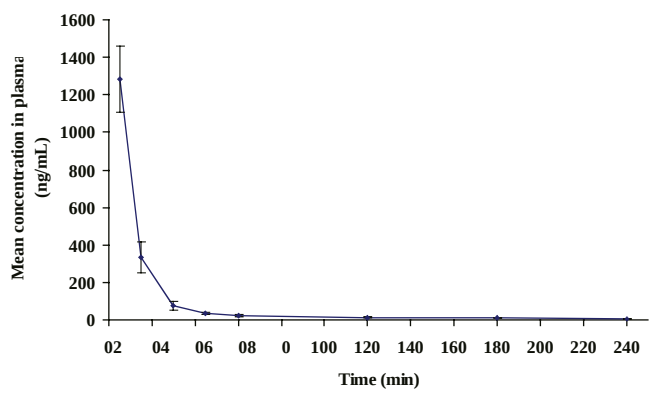

Figure 4: Mean ± S.D. plasma concentration-time profile of curcumin in rat plasma following intravenous administration of curcumin (n = 3).

Application of the method

The rat plasma samples generated following intravenous administration of curcumin 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 curcumin in rats. The mean concentration of curcumin versus the time profile is shown in Figure 4. The half-life ($t_{½}$) of curcumin was 2.29 h, while the $AUC_{(0-\infty)}$ was 429.15 ng.h/mL.

Conclusion

In conclusion, we have developed and validated a simple, specific, accurate and reproducible LC–MS/MS assay to quantify curcumin using commercially available IS from small volumes of rat plasma. 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 curcumin with desired precision and accuracy along with high throughput.

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