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Pharmacokinetic applicability of a validated liquid chromatography tandem mass spectroscopy method for orally administered curcumin loaded solid lipid nanoparticles to rats

Vandita Kakkar[a], Sukhjeet Singh[b], Dinesh Singla[b], Sudhir Sahwney[c], Anurag Singh Chauhan[c], Gangandeep Singh[c], Indu Pal Kaur[a,*]

[a] Department of Pharmaceutical Sciences, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India
[b] Formulation and Development, Panacea Biotec Ltd, Lalru 140501, India
[c] Bioanalytical Research and Development, Panacea Biotec Ltd, Lalru 140501, India

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ABSTRACT

A simple and sensitive validated LC–MS/MS analytical method was used for determination of curcumin in rat plasma, using nimesulide as internal standard. Analyses were performed on an Agilent LC–MS/MS system using a Chromolith rod^{TM} and isocratic elution with acetonitrile:10 mM ammonium acetate buffer (pH 3.5) (80:20, v/v) at a flow rate of 0.8 ml/min with a total run time of 3 min and an overall recovery of 77.15%. A triple quadrupole mass spectrometer, equipped with an electrospray ionization interface, operated in the negative mode was used. Calibration curve in plasma spiked with varying concentration of curcumin were linear over the concentration range of 10–2000 ng/ml with determination coefficient >0.99. The lower limit of quantification was 10 ng/ml. Intra and inter-day variability's (RSD) for extraction of curcumin from plasma were less than 10% and 15% respectively and accuracy was 102.43–108.5%. Multiple reaction monitoring was used to monitor the transition for curcumin (*m*/*z*; 367/217 $[M-H]^-$) and IS (*m*/*z*; 307/229). The method was applied for determining curcumin concentration in plasma after peroral administration of 50 mg/kg of free curcumin (C-S) or curcumin loaded solid lipid nanoparticles (C-SLNs) to rats. Results established selectivity and suitability of the method for pharmacokinetic studies of curcumin from C-SLNs.

1. Introduction

Curcumin (diferuloylmethane) is the active yellow pigment in turmeric, a popular plant-derived coloring spice and ingredient of many cosmetics and pharmaceuticals [1,2]. Curcumin is known to exhibit antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic activities [3].

Irrespective of the route of administration, curcumin exhibits low serum and tissue levels due to its poor solubility and absorption, extensive metabolism (intestinal and hepatic) and rapid elimination [4,5]. A study by Wahlstorm and Blenow [6] showed that 65–85% of the dose (1 g/kg) administered to Sprague–Dawley rats was excreted unchanged in the urine. Low detectable levels of radioactivity in feces was reported after oral administration of deuterium and tritium labeled curcumin [7,8]. Limiting physicochemical properties of curcumin have presented many challenges to the formulation scientist, to present it in a bioavailable form such that the clinical efficacy is enhanced. In this regard, numerous formulation strategies like nanoparticles, liposomes, microemulsion, complexation with phospholipids and cyclodextrins, solid dispersions have been taken up to improve bioavailability (BA) of curcumin [9–11].

A liquid chromatographic method with spectrophotometric detection was developed to separate and quantify curcuminoids from *Curcuma longa* L. in early fifties [12]. Another liquid chromatographic method was developed to measure curcumin and its related compounds [13]. The methods for measuring curcumin in biological samples have already been established with high-performance liquid chromatography (HPLC)-UV detection using liquid–liquid extraction from pig plasma [14]. Health et al. reported a reversed phase LC–UV system for measurement of curcumin in human plasma and urine [15]. Recently, curcumin and its metabolites were measured in biological matrices by matrix-assisted laser desorption ionization time-of-flight mass spectrometry [16]. In a very recent study UPLC method for determination of curcumin has been reported [17].

Even though quite a few methods have been developed for detecting curcumin from biomatrices, but their applicability for

* Corresponding author. Tel.: +91 172 2534191; fax: +91 172 2543101.
E-mail address: indupalkaur@yahoo.com (I.P. Kaur).

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in vivo pharmacokinetic determination after administration of formulated curcumin is lacking. In a very recent study in healthy volunteers, a 650 mg/kg dose of solid lipid curcumin particles showed plasma levels of 22.43 ng/ml, while free curcumin (95% curcuminoids extract) at the same dose was undetectable [18]. To what degree the enhanced BA is a result of increased absorption or due to reduced conversion of free curcumin to conjugates is still not clear, because in this study the samples were not pretreated with glucuronidase. Reports indicate a 2–3-fold increase in curcumin absorption when combined with different types of lipids [19].

Low detectable levels of curcumin in human biomatrices and extensive metabolism following oral dosing [3,4] suggest a need to develop a highly sensitive analytical method which would augment its clinical development. Such methods should be equally applicable for determining plasma curcumin concentration after administration of free curcumin and suitable curcumin formulations.

To the best of our knowledge, the use of liquid chromatographic technique coupled with tandem mass spectroscopy (LC–MS/MS) for the determination of curcumin *in vivo*, after oral administration of curcumin loaded solid lipid nanoparticles (C-SLNs), in rat plasma has not been demonstrated. Presently, we report a validated LC–MS/MS method and its application for determining pharmacokinetic parameters, after oral administration of free curcumin (C-S) and C-SLNs at a dose of 50 mg/kg.

2. Materials and methods

2.1. Reagents

Curcumin was a gift sample from Sanat Products Ltd, Delhi, India. The sample constituted a mixture of three curcuminoids, namely curcumin (95%), demethoxycurcumin, and bisdemethoxycurcumin (later two constitute the remaining 5%). Nimesulide was used as the internal standard (IS) and was provided by Panacea Biotec Ltd (Lalru, India). Sulfatase-free-β-glucuronidase (type IX-A from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, acetic acid, diethyl ether and other chemicals used in the preparation of buffer were purchased from Merck KGaA, Darmstadt, Germany.

All other chemicals and reagents were of analytical grade and were used without further purification.

2.2. Mass spectrometry

The liquid chromatograph (Agilent 1100; Agilent Technologies (Palo Alto, CA)) was coupled to a mass spectrometer with a turbo electrospray ion source (API 4000; LC/MS/MS triple quadrupole system, Applied Biosystems, Foster City, CA) and was used in negative ionization mode. Chromolith rod^{TM} (50 mm × 4.6 mm, 5 μm particle size; Merck KgaA, Darmstadt, Germany) kept at ambient temperature was used for the analysis. The mobile phase consisted of acetonitrile and 10 mM ammonium acetate buffer (pH 3.5) (80:20, v/v). The flow rate was 0.8 ml/min, and the total run time was 3 min.

The turbo ion-spray interface heater was maintained at 450 °C. The heating gas 1 (GS1) and heating gas 2 (GS2), generated by peak generator model NM20Z were kept at a pressure of 50 psi. The collision-activated dissociation gas pressure was 7 psi. Declustering potential was −35 V; entrance potential was −10 V; collision energy was −20 V; collision cell exit potential was −1 V. The multiple reactions monitoring pair monitored was *m*/*z* 367/217 for curcumin and *m*/*z* 307/229 for nimesulide, with a dwell time of 200 ms. The autosampler cooler was maintained at 10 °C. Analyst software (version 1.42; Applied Biosystems) was used for data interpretation and calibration. All the other statistical analysis was carried out using SigmaStat software, by one-way ANOVA.

2.3. Preparation of C-SLNs

A concentrated dispersion of curcumin loaded solid lipid nanoparticles (C-SLNs) were prepared by oil in water (o/w) microemulsification technique [20].

2.4. Animals

Wistar male rats weighing 200–300 g were used. The animals were kept under standardized conditions (with free access to food and fresh water), with a clean cage been provided twice a week. The animals were acclimatized to laboratory conditions over the week before the experiments. The experimental protocols were approved by the Institutional Animal Ethical Committee and conducted according to the guidelines by Committee for the Purpose of Control and Supervision of Experiments on Animals.

Rats were divided randomly into two groups, with 6 animals in each group, for the administrations of a single dose of free curcumin (C-S; curcumin solubilised in tween 80) and curcumin loaded solid lipid nanoparticles (C-SLNs) at a dose of 50 mg/kg in both the cases. To determine the drug concentrations and to calculate the pharmacokinetic parameters, blood samples (1 ml each) were withdrawn from sinus under clavicle at the following times: baseline (predose), 15, 30 and 45 min and 1, 2, 4, 8, 16 and 24 h after dosing. The blood samples were centrifuged at 3020 × *g* for 10 min, supernatant collected and transferred to tightly sealed plastic tubes (heparin lithium as an anticoagulant), stored at −20 °C until analysis.

2.5. Stock solutions

Concentrated stock solutions of curcumin, and IS were prepared by dissolving 10.0 mg of each in 100 ml of methanol to give 100 μg/ml stock solutions (Set I). The second set of stock solutions (Set II) was prepared as a duplicate of Set I.

2.6. Preparation of the calibration curve (CC) and quality controls (QC)

Eight point calibration curve (CC) was prepared by serial dilution of curcumin stock solution (100 μg/ml) in the range of 10–2000 ng/ml. The concentrations were corrected for potency and amount weighed. Calibration standards were prepared daily by spiking 0.1 ml of blank plasma with 10 μl of the appropriate working solution resulting in concentrations of 10, 25, 50, 125, 250, 500, 1000 and 2000 ng of curcumin per ml plasma. A plot with the resulting peak area ratios of curcumin to IS was obtained against the concentrations.

Quality control (QC) samples (low quality control (LQC), 75 ng/ml; medium quality control (MQC), 750 ng/ml; high quality control (HQC), 1250 ng/ml; lowest limit of quantification (LLOQ), 10 ng/ml) were prepared by spiking 0.1 ml aliquot of blank plasma with 10 μl of spiking solution of drug as well as the IS. All solutions were stored in the refrigerator at 5.0 ± 3.0 °C. The bulk spiked CC and QC samples were stored at −20 °C.

2.7. Sample preparation

To a 100 μl of rat plasma, 10 μl of IS, 200 μl of β-glucuronidase (for conversion of curcumin glucuronides to free curcumin) was added and the mixture was incubated at 37 °C for 1 h. Curcumin was then extracted using 2 ml of diethylether (liquid–liquid extraction; LLE) followed by vortexing (1 min) and cold centrifugation (4 °C) for 5 min at 5000 rpm. Supernatant was decanted and dried, using

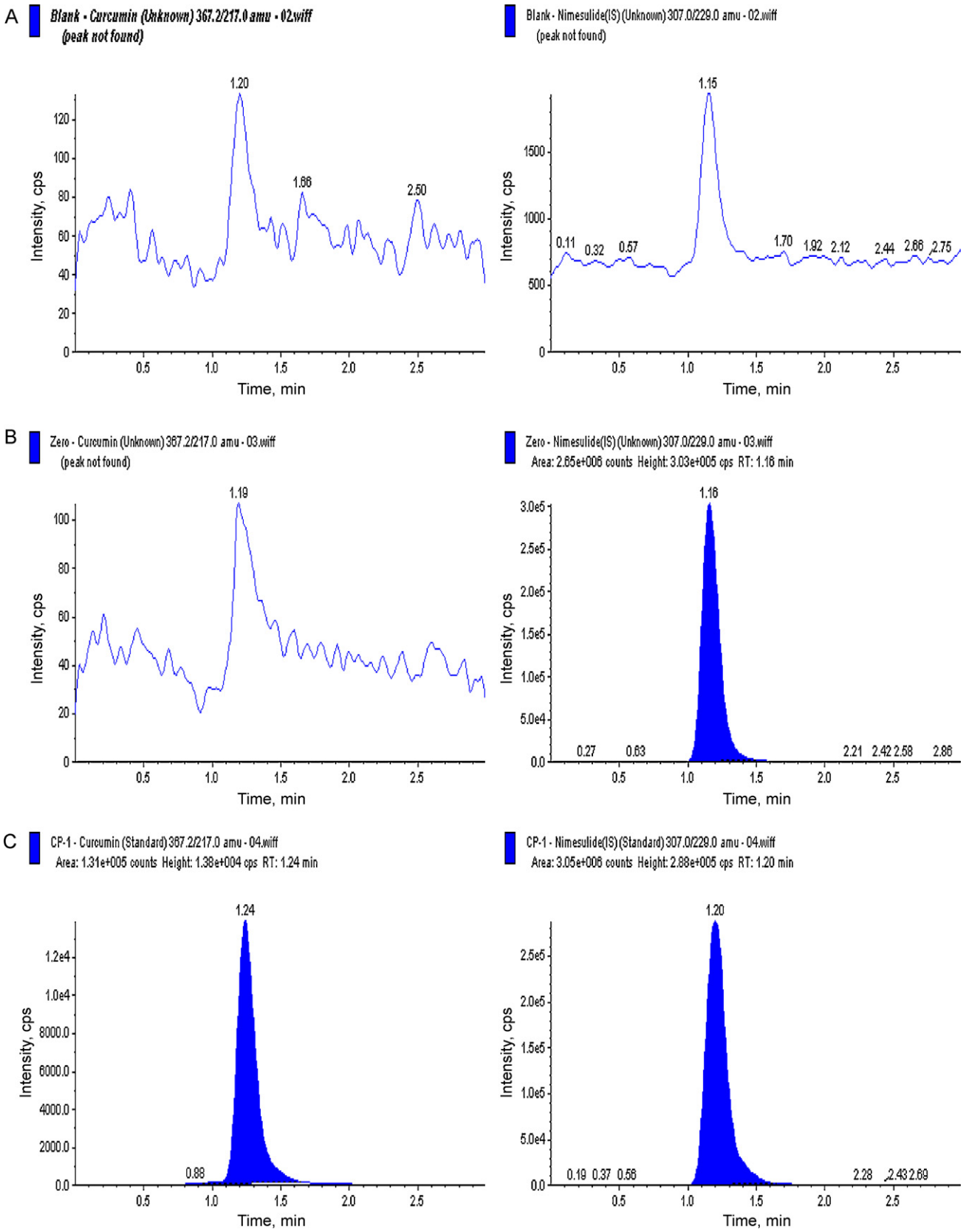

Fig. 1. Represents chromatograms of (A) blank plasma sample, (B) zero curcumin sample, and (C) curcumin in plasma. The peak on left side of the figure is representative of curcumin while that on right side are indicative of the peak of internal standard.

nitrogen gas at 50 °C, by turbo evaporator. Dry sample was reconstituted in the mobile phase and transferred to the auto sampler vials and subjected to LC–MS/MS for analysis.

2.8. Bioanalytical method validation

2.8.1. System suitability

System suitability parameters were determined by injecting seven un-extracted MQC samples before the start of each analytical run. For each system suitability average peak ratio of curcumin to IS was calculated.

2.8.2. Selectivity and specificity

A specificity exercise was performed for both methanol and plasma. Individual blank plasma samples, LLOQ-QC sample, and methanol (blank) ($n = 6$) were prepared according to the sample preparation procedure described above and screened for interference.

2.8.3. Recovery

The LLE efficiency was calculated by comparing the peak areas of extracted plasma standards with areas of reference standards added to blank plasma extract. The reference standard was prepared by extracting rat control plasma and reconstituting the evaporated extracts with stock solutions of curcumin, and IS. The concentration of the internal standard was 100 ng/ml. Recovery studies for curcumin were accomplished at three concentration levels (75, 750, and 1250 ng/ml) in rat plasma.

2.8.4. Matrix effect

To study the matrix effect, blank plasma samples were processed and spiked later to obtain MQC and HQC concentrations. The response (area) was compared with directly injected samples at MQC and HQC levels.

2.8.5. Inter-day and intra-day precision and accuracy

Inter-day and intra-day precision and accuracy were evaluated by spiking known amounts of curcumin and IS in plasma ($n = 6$). The precisions were expressed as %CV (coefficient of variation) and %accuracy was expressed by using the formula:

$$\frac{\text{Measured concentration}}{\text{Nominal concentration}} \times 100.$$

Four different concentrations (10, 75, 750 and 1250 ng/ml) were used, and samples were prepared according to the procedure as mentioned above. Intra-day precision and accuracy were assessed within one batch using replicate ($n = 6$) determinations for each concentration of the spiked plasma sample, whereas inter-day precision and accuracy was assessed on three separate occasions using replicates ($n = 6$) for each concentration used.

3. Results and discussion

A liquid chromatographic mass spectrometric method for the estimation of curcumin in rat plasma has been developed and validated according to the principles of Good Laboratory Practices. Optimization trials were carried out using sunfire C_{18}, with mobile phase acetonitrile:methanol (50:50, v/v), acetonitrile:2 mM ammonium acetate buffer (80:20, v/v), acetonitrile:10 mM ammonium acetate buffer (80:20, v/v), acetonitrile:10 mM ammonium acetate buffer (80:20, v/v) with pH adjusted with acetic acid to 3.5. The studies resulted in intense peaks but the sharpness was only moderate. Changing the column to Chromolith rod^{TM} resulted in a sharp peak with sufficient response area using acetonitrile:10 mM ammonium acetate buffer (80:20, v/v) with pH adjusted to 3.5 as mobile phase, at flow rate of 0.8 μl/ml. The method was validated over a concentration range of 10–2000 ng/ml, and the results obtained were directly obtained/extrapolated on the calibration curve. Analysis for curcumin in the prepared samples showed a retention time of 1.21 ± 0.3 min, with a total run time of 3 min. Representative chromatograms of (A) blank plasma sample, (B) zero curcumin sample, and (C) curcumin in plasma are shown in Fig. 1.

Table 1
Intra-day and inter-day precision and accuracy of curcumin in rat plasma ($n = 6$).

Nominal concentration (ng/ml)	Observed concentration (ng/ml)	%Precision	%Accuracy
Intra-day			
75	76.78 ± 1.54	2.00	102.43
750	813.67 ± 28.92	3.55	108.5
1500	1580.0 ± 43.01	2.72	105.67
Inter-day			
75	78.0 ± 2.71	3.55	108.5
750	789.42 ± 15.93	2.72	105.7
1500	1571.1 ± 34.08	2.05	106.5

The lower limit of quantization for curcumin by the developed method was found to be 10.0 ng/ml for curcumin. The between-run precision and accuracy for curcumin at 10 ng/ml were 6% and 104%, respectively. The linearity of the method was determined by a weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 10 to 2000 ng/ml.

Best-fit calibration lines of the ratio of curcumin to IS peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$. The r^2 values were consistently 0.999 during the entire course of validation. Table 1 shows a summary of intra- and inter-day precision and accuracy for curcumin in rat plasma. The intra-day accuracy of curcumin for rat plasma samples was 102.43–108.5% at QC samples with the precision (RSD) less than 3.55%. The inter-day accuracy of curcumin for rat plasma samples ranged from 105.7% to 108.5% at QC samples with the precision (RSD) less than 3.55%.

The absolute recovery of curcumin and IS was calculated for replicate spiked QC samples (LQC, MQC and HQC) (Table 2). Extraction recovery is calculated by comparing the peak area ratios of curcumin and IS respectively, in plasma samples with the peak area of curcumin and IS added to blank plasma at concentrations ranging from 75 to 1500 ng/ml. Results show an overall mean percent recovery of 77.15% for curcumin and 67.95% for IS added to blank plasma extract. It may be noted that as per the US FDA guidelines of May 2001 [21], recovery pertains to the extraction efficiency of an analytical method within the limits of variability. It is stated that recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Hence the method developed and reported presently though showing a recovery of 77.15% (for curcumin) may be considered suitable taking into account the consistency and reproducibility of the results obtained upon repetitive evaluation.

The validated method was successfully applied to measure curcumin in rat plasma and was also used to study its pharmacokinetics when administered orally in free form and as C-SLNs to rats.

Table 2
Recovery of curcumin and internal standard in rat plasma.

QC	Curcumin		Internal standard	
	%Recovery	%RSD	%Recovery	%RSD
LQC	82.91	1.77	68.88	2.21
MQC	76.71	7.09	70.95	7.49
HQC	75.13	5.53	64.01	5.52

Table 3
Pharmacokinetic parameters of C-SLN and C-S at 50 mg/kg.

Formulation	Dose (mg/kg)	$AUC_{0-\infty}$ (h μg/ml)[a]	V_d (l/kg)[a]	T_{max} (h)[a]	Cl_{obs} (l/h/kg)[a]	C_{max} (μg/ml)[a]
C-S	50	1.075 ± 0.12	41.17 ± 0.35	0.25 ± 0.00	46.50 ± 0.21	0.292 ± 0.10
C-SLN	50	41.990 ± 6.18	7.72 ± 0.43	0.5 ± 0.02	1.19 ± 0.05	14.29 ± 4.29

[a] Pharmacokinetic parameters obtained with C-S were significantly different from C-SLN at $p < 0.05$.

Developed C-SLNs had a particle size of 134 nm with an entrapment of 82% and total drug content of 94.0% [20].

The areas under the concentration versus time curves were 1075 h g/ml and 41,990 h g/ml for C-S and C-SLNs respectively, indicating a 39 times increase in BA curcumin of after oral administration of C-SLNs to rats.

Oral administration of C-S in the present study resulted in a sharp C_{max} of 0.292 μg/ml within 15 min after which the plasma concentration declined rapidly, indicating a rapid metabolism of curcumin, whereas, a relatively slow increase and sustained plasma concentration of curcumin was observed for a longer time (24 h) after the administration of a single dose of C-SLNs. A very low volume of distribution (7.72 ± 0.43 l/kg; 5.3 times lower than C-S) (Table 3) and a significantly ($p < 0.05$) high C_{max} of 14.293 μg/ml at 0.5 h with curcumin still detectable at 24 h (0.012 μg/ml), confirms the sustained effect of solid lipid nanoparticles. Values beyond the limits of detection of the developed calibration curve were determined by extrapolation. Liu et al. [19] reported a C_{max} of 0.266 μg/ml, for a 100 mg/kg dose of free curcumin (later is double the dose of C-S used by us i.e. 50 mg/kg: C_{max} – 0.292 μg/ml). Yang et al. [22] used a 10 times higher dose (500 mg/kg: C_{max} – 0.060 μg/ml) and Pan et al. [4] used a 20 times higher dose (1 g/kg: C_{max} – 0.220 μg/ml); but achieved much lower concentrations than achieved presently. It may thus, be concluded that the method used in the present study is more accurate and sensitive; however the fact that curcumin does not follow dose dependent kinetics cannot be ruled out. Further the use of tween 80 for solubilising curcumin (C-S) may also exert a penetration enhancing effect such that higher amounts of curcumin are absorbed from g.i.t. We wanted to confirm that any BA enhancement observed with C-SLNs is not solely attributable to the use of surfactants (in their preparation) that is why we used C-S as the control for comparison.

The results confirm the suitability of the developed method for determining curcumin concentration in plasma upon peroral administration of C-SLNs.

4. Conclusions

A highly validated, sensitive and specific liquid chromatographic technique coupled with tandem mass spectroscopy method for the quantitation of curcumin as such and from its prepared solid lipid nanoparticles has been developed. The method has been validated with a routine sensitivity limit of 10.0 ng/ml in 0.1 ml rat plasma. Using this method, pharmacokinetics of C-S and C-SLNs after oral administration at 50 mg/kg in rats were investigated. The plasma levels of curcumin detected after oral administration of C-SLNs in rats were much higher than ever reported by use of alternative therapeutic agents for BA enhancement of curcumin.

According to the above developed assay, the C-SLNs improved the BA of curcumin in rat significantly (39 times). Envisaging the later fact, that 50 mg/kg of SLNs could achieve 14 μg/ml which has not been achieved even with the highest dose (2 g/kg) of curcumin used in rats, we performed the pharmacokinetic studies at even lower doses (upto 1 mg/kg), to observe the % increase in plasma levels of curcumin when administered in the form of SLNs [20].

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