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Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin

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Abstract

To study pharmacokinetic properties of curcumin, a fast sensitive assay method was developed to determine curcumin and its metabolite tetrahydrocurcumin in rat plasma. The assay was based on tandem mass spectrometry detection (LC/MS/MS). Salbutamol was used as the internal standard (IS). The method had the lower limit of quantitation (LLOQ) of 0.5 ng/ml in rat plasma, which corresponds to 2.5 pg for the 5 μl injection volume. Good linearity was got to 500 ng/ml. The precision, accuracy, recovery and applicability were found to be adequate for pharmacokinetic studies.

Phospholipid complex of the natural compound curcumin was prepared in order to improve its bioavailability. Complex formation resulted in an obvious increase in bioavailability of curcumin in rat in vivo according to the assay by above LC/MS/MS method.

Keywords: LC/MS/MS; Pharmacokinetic; Curcumin; Tetrahydrocurcumin; Phospholipid complex

1. Introduction

Curcumin, the yellow pigment in *Turmeric*, is a naturally occurring polyphenolic phytochemical isolated from the powdered rhizome of the plant *Curcuma Longa* that possesses anti-inflammatory properties [1] and inhibits cancer formation in mice [2]. The National Cancer Institute is currently developing curcumin as an anticancer agent [3].

The absorption, metabolism, and tissue distribution of curcumin after oral administration of 400, 80 and 10 mg of [^{3}H] curcumin in rats [4–6] has been studied. Chuang et al. [7] showed that tetrahydrocurcumin, β-glucuronidic conjugates of curcumin and tetrahydrocurcumin are the major metabolites of curcumin in rat in vivo, and curcumin has low bioavailability in rat. The possible application of this compound in therapy is hampered by its poor absorption. Since 1980s, phospholipid complex began to be a common try to increase drugs' bioavailability since it can improve the gastrointestinal absorption to achieve higher drug concentration in plasma and lower kinetic elimination (Ke), and the technique is easy to practice by using suitable solvent treatment [8]. Several natural drugs, such as silymarin [9], dolichol [10], sapinins from Centella asiatica [11], has been found obvious bioavailability improvement through phospholipid complex formulation. In this paper, we attempted to improve the bioavailability of curcumin in vivo by preparation of its phospholipid complex and a simple method was developed to simultaneously determine curcumin and tetrahydrocurcumin in plasma after oral administration of curcumin phospholipid complex. The method requires an HPLC with mass spectrometric detection. Mass spectrometric detection with ESI offers a detection technique capable of analyzing both curcumin and tetrahydrocurcumin with high sensitivity. The combination with liquid chromatography allows the simultaneous analysis of both analytes within a 5 min run time. The precision, accuracy, recovery and applicability were proved to be good enough for pharmacokinetic studies.

According to the pharmacokinetic study data acquired, we can believe that the preparation of phospholipid complex improved curcumin's bioavailability obviously.

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2. Experimental

2.1. Materials

Curcumin (purity > 99%, by HPLC) and tetrahydrocurcumin (purity > 99%, by HPLC) were prepared in our laboratory and identified by NMR techniques, and salbutamol (purity > 99%) as internal standard were purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). Fig. 1 represents the structures of curcumin, tetrahydrocurcumin and salbutamol. A 95% soya phospholipids were purchased from Panjin pharmaceutical Co., Ltd. (Liaoning Province, China). Sulfatase-free β-glucuronidase (type IX-A from *Escherichia coli*) was purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile, acetic acid, ethyl acetate and other chemicals used in buffer system were purchased from Millipore (Milford, MA, USA). Buffer solution of pH 4 was prepared as described previously [12].

2.2. Instrumentation

The HPLC-MS/MS system consisted of an Agilent HPLC system (1100 series, USA) and a AB Sciex API 4000 tandem mass spectrometer (Applied Biosystems, USA) with (−) ESI. The analytical column C18 (Phenomenex Luna, 250 mm × 4.6 mm) was used. The mobile phase was a mixture of acetonitrile/water (70:30, v/v) with 0.005% acetic acid in concentration of 0.05 ml/l. The injection volume was 10 μl; run time was 5 min; flow rate was 0.2 ml/min. Between two consecutive pipetting, the Multiprobe needles were washed with water and methanol. Autosampler 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, tetrahydrocurcumin and salbutamol peaks in the blank sample.

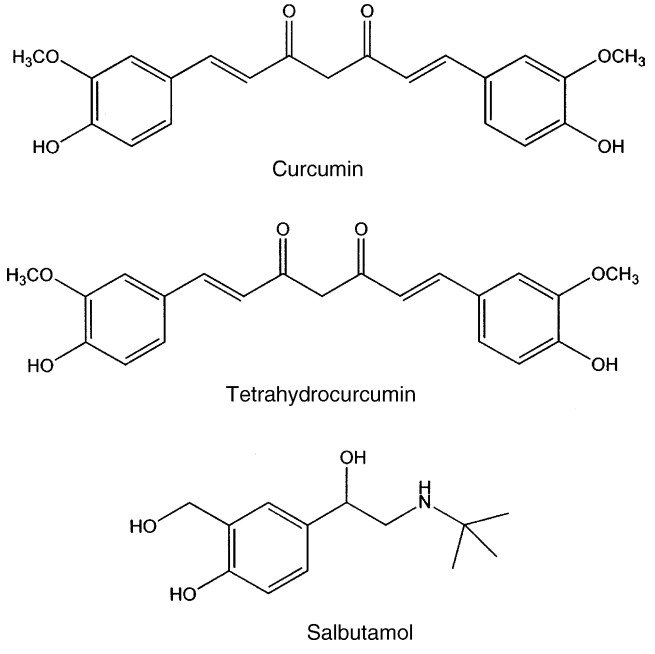

Fig. 1. Structure of curcumin, tetrahydrocurcumin and salbutamol.

The background noise in the blank sample was also not elevated. The column performance throughout the study was monitored by measuring the retention time (t_R), peak symmetry factor (B/A) at the 10% peak height where B and A were the distances after and before the peak center respectively, and the plate count (N) as 5.54 $(t_R/W_{0.5})^2$ where $W_{0.5}$ was the peak width at the half of the peak height.

The sensitivity of the multiple reactions monitoring (MRM) was optimized by testing with an infusion of 100 ng/ml curcumin and tetrahydrocurcumin solution. The ionspray needle was maintained at −4.5 kV. The turbo gas temperature was 400 °C and the auxiliary gas flow was 30 l/min. Ion source gas, curtain gas, and collision gas flows were at instrument settings of 25,14, and 30 psi, respectively. The declustering potential and focusing potential were at 46 and 200 V, respectively. The mass spectrometer was operated under MRM mode with collision energy of 24 eV. The transitions (precursor to product) monitored were m/z 373.2 → 137.1 for curcumin, 369.3 → 285.1 for tetrahydrocurcumin and 240.2 → 148.1 for salbutamol. The dwell time was 100 ms for each transition. Both quadruples were maintained at unit resolution. Chromatograms were integrated using the ANALYST version 1.3.1 software. A weighted 1/concentration 2 linear regression was used to generate calibration curves from standards and calculate the concentrations of QC samples.

2.3. Preparation of curcumin–phospholipid complex

According to the method of Pifferi Giorgio [13], 3.7 g of curcumin and 7.5 g 95% soya phospholipid were dissolved in 300 ml acetone and left under heating and stirring for 3 h. The resulting solution was concentrated. The precipitate obtained was dried in an vacuum dryer at −40 °C for 6 h. The product (10.3 g, yield 92.0%) was obtained, as a yellow opaque jell soluble in acetone.

2.4. Animals

Sprague–Dawley male rats (Shandong University) weighing 200–300 g were used. The animals were kept under standardized conditions, i.e. free access to food and water, clean cage and fresh water were provided twice a week. The animals were acclimatized to laboratory conditions over the week before the experiments.

Rats were divided randomly into two groups for the administrations of a single dose of curcumin or curcumin–phospholipid complex. To determine the drug concentrations and to calculate the pharmacokinetic parameters, eight blood samples (∼1 ml each) were drawn through the sinus under clavicle during each period at the following times: baseline (predose), 15 and 30 min and 1, 2, 6, 8 and 16 h after dosing. The blood sample was centrifuged at 3000 × g for 10 min and the supernatants of each were collected to tightly sealed plastic tubes (Heparin lithium anticoagulation), kept frozen at −20 °C until analysis.

The control blank plasma of rats were drawn through sinus under clavicle at predose 2 h, and kept frozen at −20 °C until analysis.

2.5. Stock solutions

Concentrated stock solutions of curcumin, tetrahydrocurcumin and salbutamol (IS, Internal Standard) were prepared by dissolving 5.0 mg of each in 100 ml of methanol to give 50 μg/ml stock solutions (SSI). The second set of stock solutions (SS2) was prepared as a duplicate of SSI.

2.6. Preparation of calibration standards, quality control samples and internal standard working solution

Different working solutions containing curcumin, tetrahydrocurcumin were obtained by diluting the stock solutions SSI with methanol. Calibration standards were prepared daily by spiking 1 ml of blank plasma with 25 μl of the appropriate working solution resulting in concentrations of 0.5, 50, 100, 200 and 500 ng of curcumin, tetrahydrocurcumin and salbutamol per ml plasma.

Three different concentrations of quality control samples (0.5, 200 and 500 ng/ml) were prepared by spiking 1 ml aliquots of blank plasma with 25 μl of spiking solutions freshly diluted from the stock solutions SS2.

All solutions were stored in a refrigerator at 5 ±3 °C.

2.7. Sample preparation

A 0.1 ml aliquot of plasma samples was transferred to a clean screw capped tube and then 50 μl. Phosphate buffer solution (pH 6.86; 0.1 M) with 1000U β-glucuronidase was added. The result solutions were incubated to hydrolyze the curcumin and tetrahydrocurcumin conjugates at 37 °C for 1 h. Then 50 μl of mobile phase containing 1 μl/ml of IS was added After vortexed for 30 s, 1 ml volume of ethyl acetate as extraction solvent was added and the sample was vortexed for 1 min followed ultrasonic vibrations for 15 min. After centrifugation at 15000 × *g* for 6 min. The upper organic layer was quantitatively transferred to a 5 ml glass tube and evaporated to dryness under a stream of nitrogen at room temperature. The dried extract was reconstituted in 100 μl of the mobile phase and a 10 μl aliquot was injected into chromatographic system.

2.8. Bioanalytical method validation

2.8.1. Calibration and linearity

The five-point calibration curve was constructed by plotting peak area ratio (*y*) of curcumin to the internal standard versus curcumin concentration (*x*). The regression parameters of slope, intercept and correlation coefficient were calculated by weight ($1/x^2$) linear regression in Analyst 1.3 software used in AB API4000 with weighted least squares linear regression (weighting factor $1/x^2$).

2.8.2. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, QC samples were prepared as described above. The intra-day precision of the assay was assessed by calculating the relative standard deviation (R.S.D.) for the analysis of QC samples in five replicates, and inter-day precision was determined through the analysis of QC samples on three consecutive days. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

2.8.3. Recovery

The liquid–liquid extraction efficiency was calculated by comparing the peak areas of extracted plasma standards with areas of reference standards added to blank plasma extract. The reference standards were prepared by extracting rat control plasma and reconstituting the evaporated extracts with stock solutions of curcumin, tetrahydrocurcumin and internal standards. The concentration of the internal standards was 100 ng/ml. The recovery study for curcumin was accomplished at three concentration levels (0.5, 200, and 500 ng/ml) in rat plasma.

2.8.4. Stability

Bench-top stability was assessed by leaving the QC samples at three different concentrations at room temperature for 4 h. Freeze/thaw stability was assessed over three cycles. QC samples at three different concentrations were thawed at room temperature and refrozen at −20 °C over three cycles and assayed. The stability of reconstituted samples in autosampler vials was assessed at ambient temperature for over 24 h. The freezer storage stability of curcumin and tetrahydrocurcumin in rat plasma at −20 °C was evaluated by assaying QC samples at beginning and 4 weeks later. Freshly processed standard samples were used to quantitate all the QC samples. All stability QC samples were analyzed in triplicate.

2.8.5. Matrix effect

The matrix effect was studied by analyzing standards of curcumin and tetrahydrocurcumin injected directly in mobile phase, and standards spiked into extracts of blank plasma in the presence or absence of phospholipid equivalent to the content in complex. The response (peak area) of curcumin and tetrahydrocurcumin of each group was compared.

2.9. Pharmacokinetics analysis

Since tetrahydrocurcumin, glucuronide conjugates of curcumin and tetrahydrocurcumin are major metabolites of curcumin in vivo [7], to estimate objectively the bioavailability of the two drugs, the conjugates described above were first hydrolysed [14], and tetrahydrocurcumin plasma concentration was added to curcumin plasma concentration through converting according to the formula $C_{\text{curcumin}} = C_{\text{tetrahydrocurcumin}} \times (368/372)$ to obtain the total curcumin absorption concentration in rats. Pharmacokinetic parameters were calculated from the total drug plasma concentration curve against the real time of extraction for each rat using an Application 3P97 (Chinese Pharmacological Society). The relative bioavailability of curcumin–phospholipid complex is calculated by formula $F = \text{AUC}_{\text{complex}}/\text{AUC}_{\text{curcumin}} \times 100\%$.

3. Results

3.1. Mass spectrum

Mass spectrum of curcumin $[M+H]^+$ m/z 369, tetrahydrocurcumin $[M+H]^+$ m/z 373 and the internal standard salbutamol $[M+H]^+$ m/z 240 are shown in Fig. 2.

3.2. Chromatography and specificity

Under optimized HPLC and MS conditions, curcumin, tetrahydrocurcumin and the internal standard were detected respectively (Fig. 3), and MS revealed a double peak with tetrahydrocurcumin, such phenomenon reflected rapid transition between the keto–enol β-diketone molecular species of

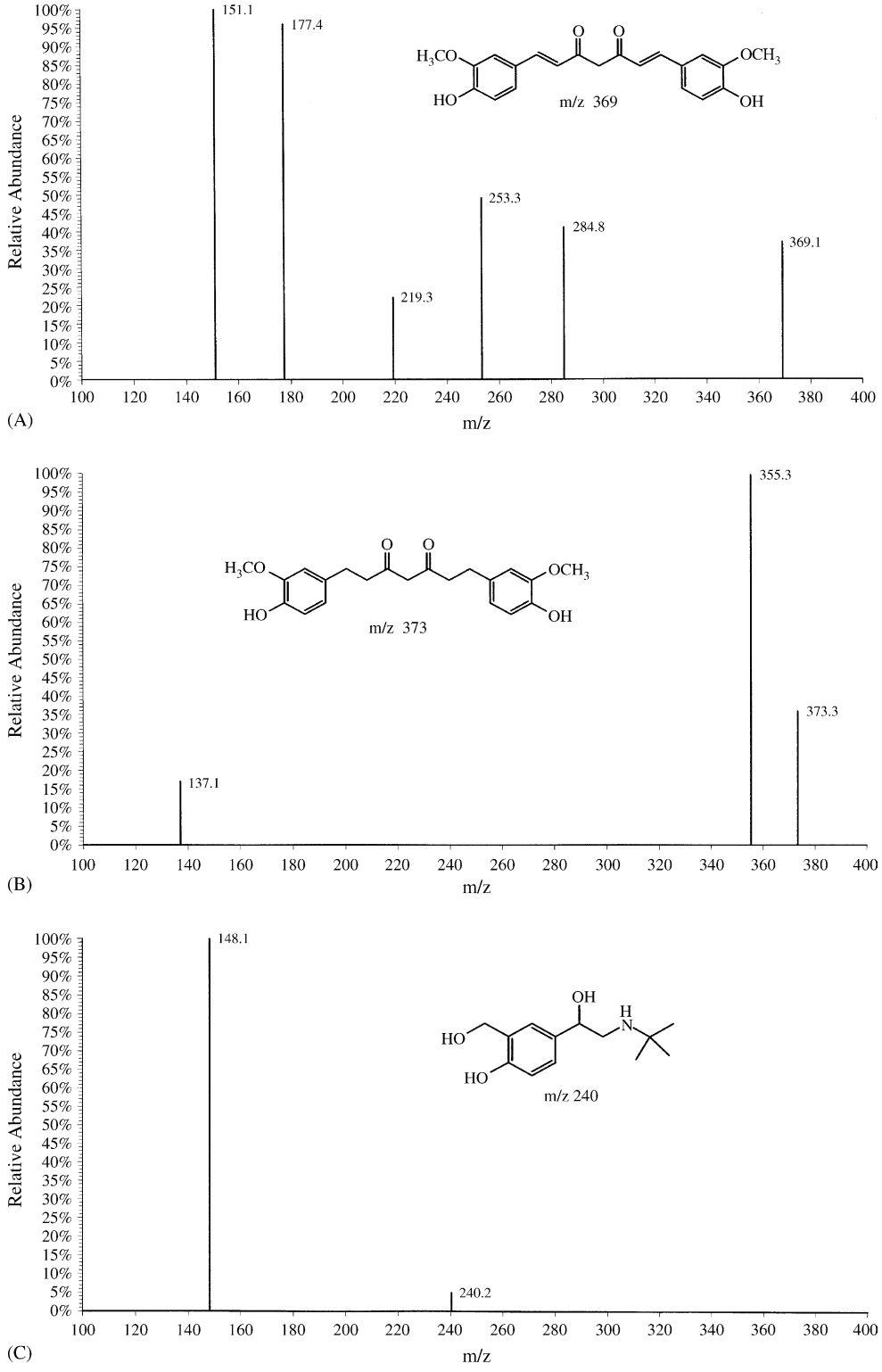

Fig. 2. Product spectrums of curcumin (A), tetrahydrocurcumin (B) and salbutamol (C).

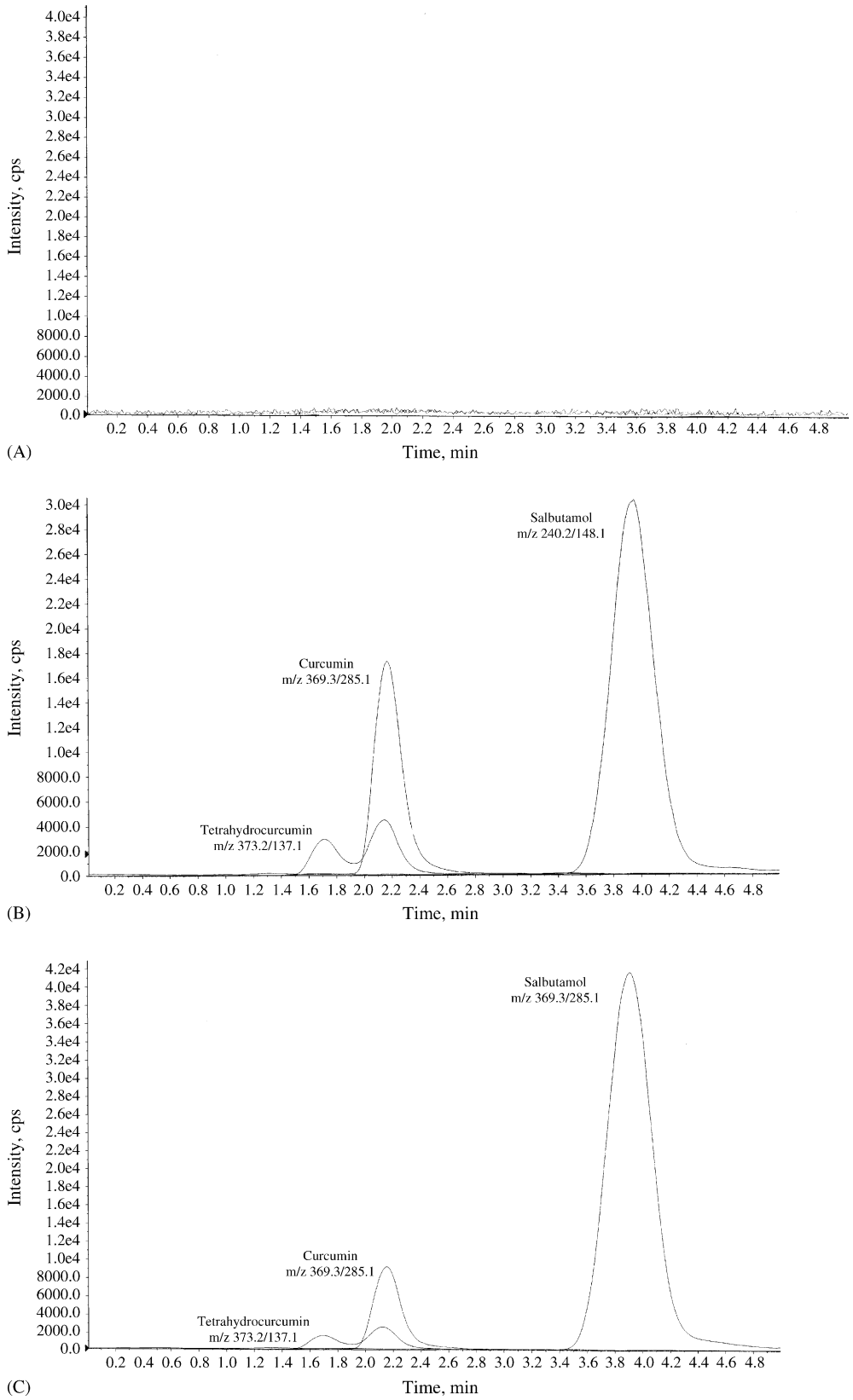

Fig. 3. MRM spectrum of curcumin, tetrahydrocurcumin and the internal standard salbutamol: (A) blank rat plasma, (B) blank rat plasma with curcumin, tetrahydrocurcumin and salbutamol, and (C) rat plasma after oral administrated curcumin for 2 h.

Table 1
Precision and accuracy of LC/MS/MS analysis of curcumin and tetrahydrocurcumin in rat plasma

Nominal concentration (ng/ml)	Curcumin			Tetrahydrocurcumin		
	Measured (mean ± S.D.)	Precision R.S.D.%	Accuracy[a] DEV%	Measured (mean ± S.D.)	Precision R.S.D.%	Accuracy[a] DEV%
Intra-day ($n = 5$)						
0.5	0.47 ± 0.06	13.20	−6.00	0.48 ± 0.07	13.75	4.32
200	195.60 ± 11.69	5.98	−2.20	202.61 ± 2.13	5.98	1.30
500	489.96 ± 12.54	2.56	−2.01	479.23 ± 30.46	6.36	4.15
Inter-day ($n = 15$)						
0.5	0.45 ± 0.05	12.00	−9.52	0.47 ± 0.06	12.47	−5.05
200	194.46 ± 8.52	4.38	−2.77	202.96 ± 13.76	6.78	1.48
500	486.65 ± 18.33	3.77	−2.67	488.05 ± 19.06	3.91	−2.39

[a] Accuracy defined as [(measured concentration − added concentration)/added concentration] × 100%.

tetrahydrocurcumin as published before [13], the single tautomer of tetrahydrocurcumin cannot be obtained, but in different conditions (tetrahydrocurcumin concentration, temperature and mobile phases), both of them had same MS spectra, in accord with the studies of Yoshifumi Akama et al. [15], so the sum of both peak area was used to represent the quantity of tetrahydrocurcumin. Since no late-eluting peaks were observed, regeneration of the column using a gradient elution step was not necessary. The total run time was 5 min and much shorter than the previously published method [2]. Blank rat plasma showed no significant interfering peaks at the retention times of curcumin, tetrahydrocurcumin and the internal standards (Fig. 3).

3.3. Calibration and linearity

The calibration curve was linearity over the concentration range of 0.5–500 ng/ml of curcumin and tetrahydrocurcumin in rat plasma with correlation coefficients $r = 0.9962$ and 0.9968 respectively and consistent slope values when evaluated by weighted ($1/x^2$) Linear regression. The calibration curve of curcumin and tetrahydrocurcumin is $Y = 0.0266X + 0.00472$ and $Y = 0.0129X + 0.00252$, respectively.

Table 2
Recovery of curcumin and tetrahydrocurcumin in rat plasma

Concentration (ng/ml)	Curcumin recovery ($n = 6$) (%)	Tetrahydrocurcumin recovery ($n = 6$) (%)
0.5	72.35	75.21
200	83.27	80.07
500	75.82	79.19

3.4. Precision and accuracy

Table 1 shows a summary of intra- and inter-day precision and accuracy for curcumin and tetrahydrocurcumin in rat plasma. The intra-day accuracy of curcumin and tetrahydrocurcumin for rat plasma samples was 94.0–98.0% and 95.7–101.3% at QC samples with the precision (R.S.D.) less than 13.75% and 2.56%, respectively. The inter-day accuracy of curcumin and tetrahydrocurcumin for rat plasma samples ranged from 90.5% to 97.3% and from 95.0% to 101.5% at QC samples with the precision (R.S.D.) less than 12.47% and 3.77%, respectively. So, it is expected that the present method will be applicable to the metabolite kinetics of curcumin for rat.

3.5. Recovery

Extraction recovery is calculated by comparing the peak area ratios of curcumin and tetrahydrocurcumin in plasma samples with the peak area ratios of curcumin and tetrahydrocurcumin added to blank plasma extract. The recoveries for curcumin and

Table 3
Stability of curcumin and tetrahydrocurcumin in rat plasma

Sample condition	Nominal concentration (ng/ml)											
	Curcumin						Tetrahydrocurcumin					
	0.5		200		500		0.5		200		500	
	Observed%	DEV	Observed%	DEV	Observed%	DEV	Observed%	DEV	Observed%	DEV	Observed%	DEV
Freshly prepared	0.49	2.8	208.35	4.2	487.25	2.6	0.51	2.4	193.62	3.2	483.15	3.4
4 h at room temperature	0.47	5.4	195.33	2.3	458.38	8.3	0.45	9.6	185.62	7.2	534.15	6.8
Autosampler 24 h stability	0.46	8.8	185.365	7.3	469.572	6.1	0.46	7.6	192.15	3.9	476.52	4.7
4 weeks at 20 °C	0.48	3.6	195.356	2.3	463.251	7.3	0.47	5.6	205.32	2.7	452.65	9.5
Freeze/thaw cycle no. 1	0.47	7.0	192.151	3.9	443.65	11.3	0.46	8.2	187.15	6.4	465.15	7.0
Freeze/thaw cycle no. 2	0.47	5.6	194.325	2.8	445.356	10.9	0.48	3.6	179.25	10.4	467.18	6.6
Freeze/thaw cycle no. 3	0.46	7.8	189.325	5.3	479.215	4.2	0.46	7.6	184.36	7.8	457.15	8.6

All QC samples were analyzed in triplicate. Mean values are reported.

Table 4
Matrix effect of plasma for curcumin and tetrahydrocurcumin

Observed concentration			
Curcumin dissolved in mobile phase	0.50 ± 0.01	200.30 ± 0.50	501.91 ± 2.02
Blank plasma extraction spiked with curcumin	0.52 ± 0.01	203.13 ± 2.12	516.01 ± 9.37
Matrix effect	3.97%	0.95%	3.17%
Blank plasma with phospholipid[a] extraction spiked with curcumin	0.52 ± 0.01	211.15 ± 5.75	582.19 ± 25.08
Matrix effect	2.65%	4.93%	5.61%
Tetrahydrocurcumin dissolved in mobile phase	0.50 ± 0.01	201.22 ± 0.51	500.13 ± 1.15
Blank plasma extraction spiked with tetrahydrocurcumin	0.53 ± 0.01	209.45 ± 7.49	519.21 ± 10.68
Matrix effect	5.96%	4.57%	3.45%
Blank plasma with phospholipid[a] extraction spiked with tetrahydrocurcumin	0.55 ± 0.02	208.28 ± 3.05	520.73 ± 9.00
Matrix effect	9.27%	3.98%	3.75%

[a] Blank plasma with phospholipid were prepared by adding corresponding phospholipid to curcumin–phospholipid complex by molar ratio.

tetrahydrocurcumin from 0.5 to 500 ng/ml are listed in Table 2 and show an overall mean percent recovery of 77.15% for curcumin and 78.16% for tetrahydrocurcumin.

3.6. Stability

No significant loss of curcumin (≤8.3%) and tetrahydrocurcumin (≤9.6%) was observed after storage of plasma at room temperature on the bench-top for at least 4 h (Table 3). Processed samples were stable up to 24 h in the autosampler tray (Table 3). Plasma samples were stable at −20 °C for at least 4 weeks with no significant loss of curcumin (≤7.3%, Table 3) and tetrahydrocurcumin (≤9.5%, Table 3). Plasma samples were stable over at least three freeze/thaw cycles (Table 3).

3.7. Matrix effect

Matrix effect was evaluated during method development of the simultaneous analysis of curcumin and tetrahydrocurcumin in rat plasma (see Table 4). The overall mean matrix effect value of blank plasma is 2.70% for curcumin and 4.66% for tetrahydrocurcumin, and the over all mean matrix effect of plasma with phospholipid is 4.40% for curcumin and 5.67% for tetrahydrocurcumin. Matrix effect showed little influence for the quantitation of curcumin and tetrahydrocurcumin in every group and can be omitted. So this method works for plasma with curcumin or curcumin–phospholipid complex.

3.8. Pharmaco kinetic parameters

The curcumin and curcumin phospholipid was administered to rats via oral routes by a dose of 100 and 300 mg, respectively. Curcumin–phospholipid complex showed very high plasma concentrations, high clearance and long half-life in rats. Our study has shown that maximum plasma drug concentration (C_{max}, sum of curcumin and tetrahydrocurcumin) is 266.70 ng/ml with T_{max} = 1.62 h. In contrast, the C_{max} for phospholipid complex of curcumin is 600.93 ng/ml with T_{max} = 2.33 h. The $AUC_{0-\infty}$ (ng min/ml) of curcumin after oral administrated with curcumin and phospholipid complex of curcumin is 2609.04 and 8772.57, respectively. This results have shown that the bioavailability have been improved obviously after oral administration with phospholipid complex of curcumin. The main pharmacokinetics parameters of two drugs in rat are shown in Table 5, and the drug concentration–time profile is shown in Fig. 4.

Table 5
Comparative analysis of the pharmacokinetic parameters

Parameters	Curcumin	Curcumin phospholipid complex
α (h^{-1})	2.13 ± 1.59	1.35 ± 0.85
β (h^{-1})	0.12 ± 0.05	0.08 ± 0.03
$t_{1/2\alpha}$ (h)	0.33	0.51
$t_{1/2\beta}$ (h)	5.7	8.44
T_{max} (h)	1.62	2.33
C_{max} (ng/ml)	266.7	600.93
$AUC_{0-\infty}$ (ng/ml h)	2609.04	8772.57

T_{max} = time to maximum plasma drug concentration (C_{max}); $AUC_{0-\infty}$ = measured value plus extrapolation from 3P97; $t_{1/2}$ = half-life.

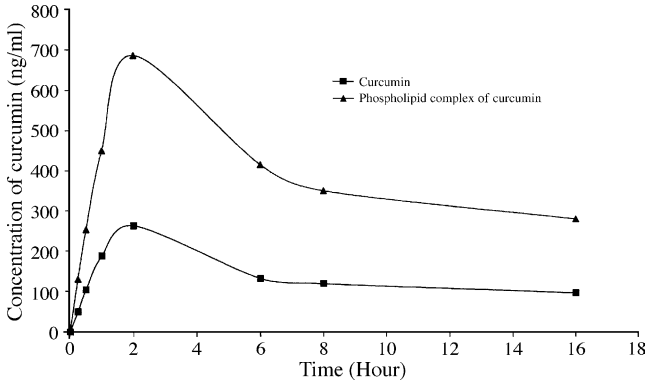

Fig. 4. Representative plasma concentration–time profiles of curcumin and curcumin phospholipid complex in rats following its oral administration at 100 and 300 mg/kg.

4. Discussion

The method is generally highly sensitive and specific, and it simplify the sample cleanup. In this method, the elution time can be kept short that makes the sample analysis very efficient, no appreciable ion suppression was found in MRM transitions of m/z 373.2 → 137.1 and 240.2 → 148.1 for curcumin and tetrahydrocurcumin, respectively, when the ethyl acetate extraction residue of rat plasma was spiked with the analytes.

Later, we were able to shorten the retention time to about 4.5 min with some degree of peak overlap between curcumin and tetrahydrocurcumin without compromising the specificity of the detection and quality of the results.

The phenomenon of double peak for tetrahydrocurcumin is inevitable, so the peak areas was added together to present then quantity of tetrahydrocurcumin. Good linearity, precision and accuracy for tetrahydrocurcumin proved the feasibility of our processing in this problem.

In our studies, phospholipid can highly increase the bioavailability of curcumin in rats via oral routes.

5. Conclusion

A highly sensitive and specific LC/MS/MS method for the quantitation of curcumin has been developed. The method has been validated with a routine sensitivity limit of 0.5 ng/ml in 0.1 ml rat plasma. Using this method, pharmacokinetics of curcumin and curcumin phospholipid complex at 100 and 300 mg/kg respectively following the oral route in the rat was investigated. The plasma levels of the two drugs have been detected at lower level and shorter duration than previously documented.

According to our assay by above method, the phospholipid complex of curcumin improved the availability of curcumin in rat significantly. This would be more helpful to make curcumin a better anticancer drug. Further studies are required to evaluate the advantages of phospholipid complex of curcumin.

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