

Research Article

Validated HPLC–MS/MS Method for Simultaneous Determination of Curcumin and Piperine in Human Plasma

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Abstract

Purpose: To develop a sensitive and rapid method for the simultaneous determination of curcumin and piperine in human plasma.

Methods: The method was based on high-performance liquid chromatography (HPLC) with electrospray ionization tandem mass spectrometer (ESI-MS/MS) detection in positive ionization mode. The analytes and internal standard were isolated from acidified plasma using liquid–liquid extraction (LLE). The organic extracts were evaporated, reconstituted in mobile phase and injected into the HPLC-MS/MS system. The analytes were chromatographed on a XB-C8 analytical column and MS-MS detection was performed on an electrospray ionization tandem mass spectrometer operated in multiple reaction monitoring (MRM) mode. Precursor→product combinations of m/z 369.9→177.0, 286.3→201.1 and 285.6→193.1 were used to quantify curcumin, piperine and the internal standard (IS), respectively. The assay was validated in the concentration range of 1.0 – 100.0 ng/ml for curcumin and 0.5 – 800.0 ng/ml for piperine using 0.5 ml of plasma.

Results: The lowest limit of quantification (LLOQ) for curcumin and piperine was 1.00 and 0.50 ng/ml, respectively. The precision of the assay (expressed as coefficient of variation, CV) was < 12.6 % at all concentrations within the standard curve range, with adequate assay accuracy. Stability data revealed that the drugs were stable in plasma under various test conditions.

Conclusion: The method is highly selective and rugged for the estimation of curcumin and piperine in human plasma and would be applicable to toxicokinetic, pharmacokinetic, bioavailability, and bioequivalence studies.

Keywords: Curcumin, Piperine; HPLC-MS/MS, Simultaneous determination

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INTRODUCTION

Curcumin[1,7-bis(4-hydroxy-3-ethoxyphenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane], is the major constituent of the yellow spice, turmeric, derived from the rhizomes of *Curcuma spp.*, which has been reported to have a variety of biological activities and pharmacological actions, such as anti-tumor, anti-inflammatory, anti-virus, anti-oxidation and anti-HIV, and low toxicity with promising clinical applications [1, 2]. However, curcumin is slightly absorbed in the gastrointestinal tract (GIT) due to its rapid metabolism by conjugation and reduction, and its oral bioavailability is very low (only 1 % in rats) [1, 2]. Pharmacokinetic properties of curcumin indicate that following oral administration, it is poorly absorbed [1] and only traces of the compound appear in the blood, while most of it is excreted in the faeces [1]. Piperine, the major alkaloidal component of *Piper longum* and *Piper nigrum*, has been reported to enhance the bioavailability of curcumin by 2000 % in human volunteers [1].

In view of the potential pharmacological effects of curcumin and the possible role of piperine in the enhancement of its bioavailability, the simultaneous determination of these drugs in biological matrices can assist in monitoring their effectiveness, treatment compliance as well as help prevent adverse events and formulate optimum dosages.

Several methods have been developed to quantify curcumin and piperine individually, mainly using HPLC with UV [1-7] and MS detectors [1]. A reversed phase HPLC method has been reported for simultaneous determination of curcumin and piperine in human plasma [1]. However, the LOQs derived from the methods above were not low enough to enable pharmacokinetic study, and there has been no report of their simultaneous determination by HPLC-MS/MS. Due to ever increasing demands for assays with higher sensitivity, we have developed successfully a more sensitive

liquid chromatography (LC)/tandem mass spectrometry (MS/MS) method with a lower limit of quantitation (LLOQ) of 1.00 ng/ml for curcumin and 0.50 ng/ml for piperine.

The chemical structures of curcumin, piperine and the IS used (diazepam) are shown in Fig 1.

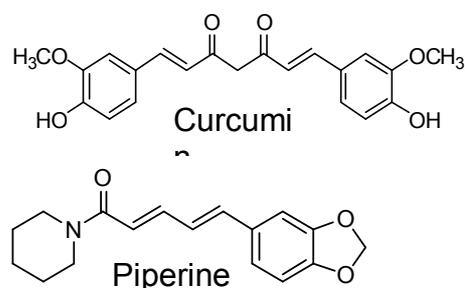


Fig 1: Chemical structures of curcumin, piperine and diazepam (IS)

EXPERIMENTAL

Chemicals and reagents

Reference standards of curcumin, piperine and diazepam (IS) with purity greater than 95% were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sulfatase-free β -glucuronidase (type IX-A from *Escherichia coli*) was purchased from Sigma (St. Louis, MO). HPLC-grade acetonitrile, methyl t-butyl ether (MTBE), acetic acid, ammonium acetate and formic acid (99 %) were purchased from Tedia Company, Inc, Fairfield, USA.

LC-MS/MS conditions

An LC system (Waters Corporation, Milford, Massachusetts) consisting of an Acquity High Performance LC and electrospray ionization tandem mass spectrometer (ESI-MS/MS; Quattro Premier XE, Waters Corporation) was used. ESI-MS/MS was performed in the positive mode: capillary and cone voltages were 3.0 kV and 35 V, respectively; drying

gas (N₂) flow rate was 750 L/h; the ionization sources were 120 °C; and the desolvation temperature was 400 °C. Multiple-reaction monitoring, using the precursor → product combination of m/z 369.9→177.0, m/z 286.3→201.1, m/z 285.6→193.1 was used to quantify curcumin, piperine and the internal standard (IS, diazepam), respectively. The samples were separated on XB-C8 column (4.6 × 250 mm, 5µm, Welch Materials, Inc, Maryland, USA) using acetonitrile/formic acid (0.15%) (60:40, v/v) as the mobile phase. The flow rate was 1.0 ml/min, and post-column splitting ratio was 4:1.

Standard solutions

Curcumin is not stable when pH is greater than 7.0 [1]. Therefore, its stock solution (50.0 µg/ml) was prepared in acetonitrile-ammonium acetate buffer (pH 4.5, 0.1M, 50:50, v/v). This solution was further diluted with the buffer to a series of working standard solutions (25.0, 125.0 and 1250.0 ng/ml, respectively). The stock solution of piperine was prepared in acetonitrile–water (50:50, v/v), and further diluted to give a series of working standards (25.0, 250.0, and 2500.0 ng/ml, respectively) in acetonitrile–water (50:50, v/v). The internal standard (IS, diazepam) stock solution (100.0 µg/ml) was prepared and further diluted to produce an IS working solution (1.0 µg/ml) using acetonitrile–water (50:50, v/v). All solutions were prepared in amber glassware and stored at 4 °C. Due to stability considerations, all working standard solutions were prepared from the primary stock solution every month.

Preparation of calibration standards and test samples

Calibration standards were prepared by adding the appropriate volume of each working standard to human control plasma to obtain a final volume of 500 µl, giving seven calibration standards in the range 1.0 - 100.0 ng/ml for curcumin and eight in the range 0.5 - 800.0 ng/ml for piperine. Test samples were prepared in the same way as the calibration

standard samples, with the test samples at final concentrations of 2.0, 10.0 and 80.0 ng/ml for curcumin, and 2.0, 30.0 and 500.0 ng/ml for piperine. All test samples were stored at -70 °C until assayed.

Several aliquots of the three fractions were stored at -70 °C as controls for future assays and used to check short-term stability under storage conditions. The analytes were considered stable at each concentration when the difference between the freshly prepared samples and the stability testing samples is not more than 15 % of the nominal concentration.

Sample preparation

The frozen plasma samples were thawed at room temperature, vortexed and centrifuged at 12000 rpm for 5 min prior to preparation; 500 µl of it was aliquoted into a 10-ml centrifuge tube and then 100 µl of phosphate buffer solution (75 mM; pH 6.8) with 1000U β-glucuronidase was added. The resulting solution was incubated to hydrolyze the curcumin conjugates at 37 °C for 1 h. Following incubation, the samples were spiked with 100 µl of 0.1M ammonium acetate buffer (pH 4.5) and 30 µl of IS working solution and then extracted with methyl-t-butyl ether (5 ml) by rotating mixing. The organic extract was transferred to another clean 10 ml centrifuge tube and evaporated under a stream of nitrogen at 40 °C. The dried residue was reconstituted in 100 µl mobile phase. The reconstituted samples were vortexed for 30 s and centrifuged at 3000 rpm for 5 min, then 10 µl of the supernatant was injected into the HPLC-MS/MS system.

Validation study

The methods were validated for selectivity, linearity, intra- and inter-day accuracy, and precision. LLOQ, matrix effect, stability of the analytes in biological matrix during short- and long-term storage and after three freeze–thaw cycles were also tested.

Selectivity of the methods was investigated by analysing individual blank plasma samples of six donors for endogen interference with the analytes. Five calibration standards at LLOQ were used as reference samples.

The peak-area ratios of analyte/IS versus the nominal concentrations were plotted and a least-squares linear regression weighted by the reciprocal of the concentration ($1/x$) was applied to generate the calibration curve. Test sample concentrations were calculated from the regression equation.

LLOQ was defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision ($\pm 20\%$) and accuracy (range 80 – 120 %) [1]. Precision and accuracy (i.e., ratio of mean actual concentration : theoretical concentration expressed as a percentage) were evaluated on three different days by determining the analytes in five replicates of test samples at nominal concentrations of 2.0, 10.0 and 80.0 ng/ml for curcumin, and 2.0, 30.0 and 500.0 ng/ml for piperine. A standard calibration curve was prepared and processed each day to analyse the test samples.

The precision of the method at each concentration was reported as coefficient of variation (CV %), i.e., expressing standard deviation as a percentage of the mean calculated concentration, while the accuracy of the measure was determined by expressing the mean calculated concentration as percentage of the nominal concentration.

Extraction recovery (%) of the two analytes was determined in five replicates and at three plasma concentrations of the test samples, and estimated by comparing peak-area ratios of both analytes from extracted plasma samples to those from prepared standards.

Plasma matrix effect was tested by comparing the chromatographic peak areas from neat standards and blank plasma extracts spiked with neat standards at three

concentrations of test samples for curcumin and piperine. The stability of the compounds was evaluated by analysing test samples in triplicate at each concentration level, immediately after preparation and after leaving 12 h at room temperature or after three freeze–thaw cycles in plasma, as well as after approximately 2 months of storage at $-70\text{ }^{\circ}\text{C}$. In each case, the analytes were quantified in the stored samples using a freshly prepared calibration curve.

During all the operations, samples were protected from light due to the susceptibility of curcumin and piperine to photodegradation [**Error! Bookmark not defined.**,1].

Human studies

Nine healthy Chinese volunteers were recruited. The study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (Changsha, China) and informed consent was obtained from all subjects. The volunteers were instructed not to take any drug one week before and during the trials. On the trial day, after an 8 h overnight fast, 600 mg of curcumin plus 20 mg of piperine were co-administered and blood samples drawn at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48 and 60 h after dosing. Each blood sample was collected in a heparinized tube and immediately centrifuged. The separated plasma was stored frozen at $-70\text{ }^{\circ}\text{C}$ until analyzed.

Data analysis

Data are reported as mean \pm standard deviation (SD) for the replicates. The CV (or RSD) values and accuracy were calculated using Microsoft Excel 2003.

RESULTS

LC-ESI-MS/MS analyses

The analytes were detected in positive ionization mode by monitoring their

precursor-product combination in multiple reaction monitoring (MRM) mode. The detecting channels were m/z 369.9→177.0 m/z for curcumin (channel 2), m/z 286.3→201.1 m/z for piperine (channel 1) and m/z 285.6→193.1 m/z for the IS (channel 3).

The product scan spectra for the protonated molecule $[M+H]^+$ of curcumin, piperine and the IS are shown in Fig 2.

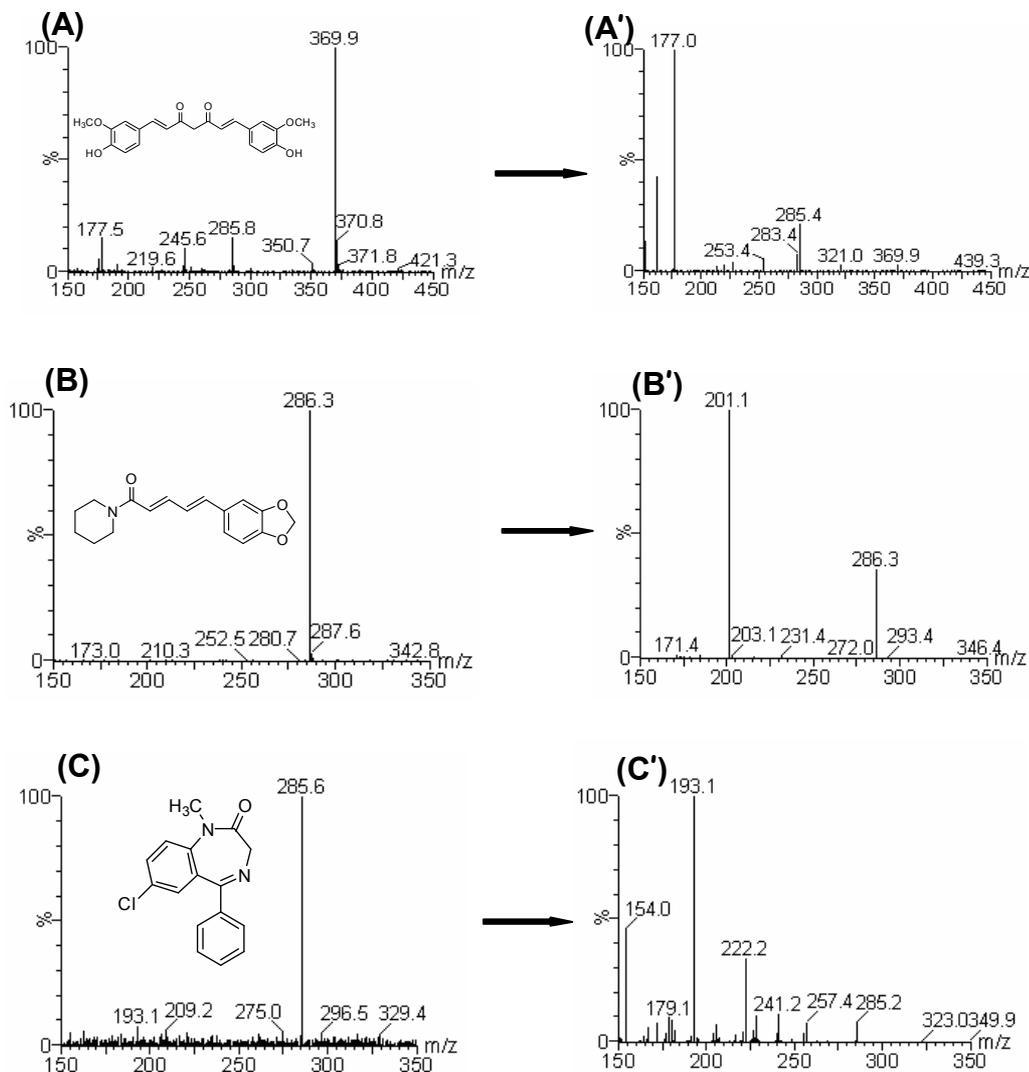


Fig 2: The positive ion ESI-MS/MS spectra of curcumin (A), piperine (B) and internal standard (C), and their chemical structures

Selectivity

The elution of the analytes was selective with a good separation of peaks in about 8 min (curcumin at 5.90 min, piperine 6.64 min and

IS 6.33 min). Fig 3 shows the representative LC-MS/MS chromatograms of blank plasma (A), blank plasma spiked with curcumin and piperine at LLOQ (B), and human plasma

sample taken 6 h after oral administration of the drugs (C).

Calibration curves and LLOQ

LLOQ, or sensitivity, is defined as the lowest concentration on the standard curve that can be measured with acceptable precision ($\leq 20\%$) and accuracy ($\leq 20\%$ of the nominal value)[18]. Based on this method, LLOQ for curcumin and piperine were 1.0 and 0.5 ng/ml, respectively.

The calibration curves showed good linearity and acceptable data over a wide range of concentrations (1.0 - 100.0 ng/ml for curcumin, and 0.50 - 800.0 ng/ml for piperine), with regression coefficient (R^2) ≥ 0.998 . Mean accuracy was in the range 81.3 - 119.9 % (at LLOQ) for curcumin and between 98.1 and 116.6 % for piperine. Precision, expressed as CV %, was in the range 2.91 - 13.10 % for curcumin and 0.51 - 11.21 % for piperine.

Precision and accuracy

The accuracy and precision for the analytes are shown in Table 1. The method was precise and accurate, with intra- and inter-day CV $\leq 12.36\%$ and accuracy in the range of 94.40 - 108.36 % for curcumin and 94.60 - 103.82 % for piperine.

Matrix effect

The results show that the peak area ratio of spiked plasma sample to their corresponding neat solution ranged from 101.99 to 106.81% for both compounds.

Recovery

Mean extraction recovery for curcumin at 2.0, 10.0 and 80.0 ng/ml was $\geq 85.6\%$, with good reproducibility indicated by CV% in the range 2.44 - 6.86 %. Recovery of piperine at 2.0, 30.0 and 500.0 ng/ml was $> 87.4\%$ with reproducibility expressed as CV $\leq 7.45\%$.

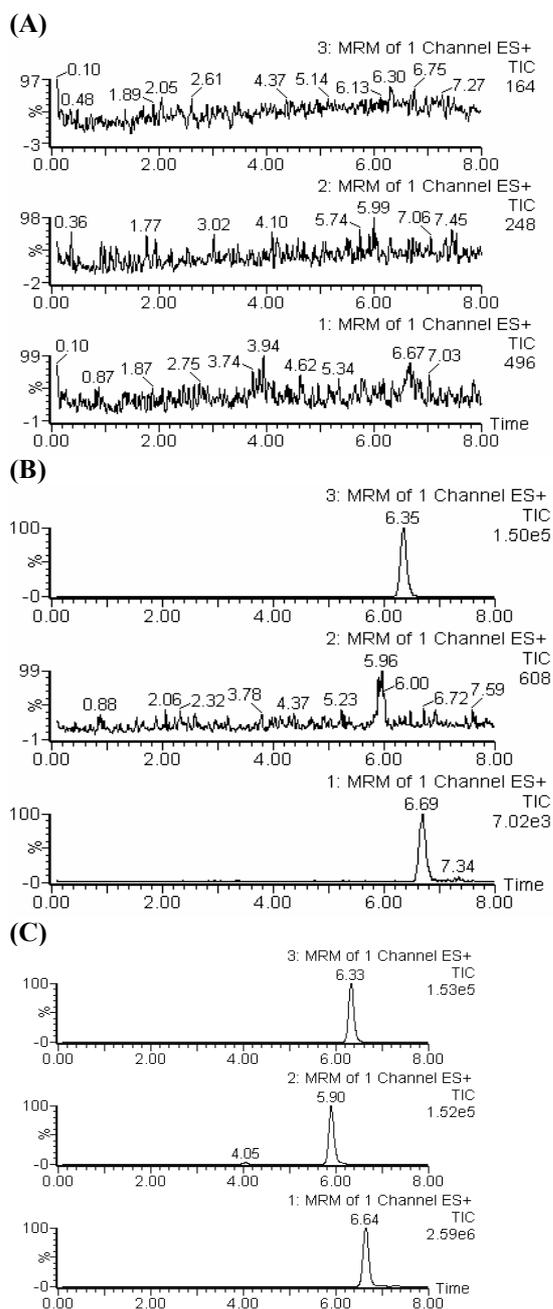


Fig 3: Representative chromatograms of (A) blank plasma; (B) plasma spiked with curcumin and piperine at LLOQ; (C) human plasma sample taken 6 h after the oral treatment with the drugs. *Note:* Piperine = channel 1; curcumin = channel 2; diazepam = channel 3

Table 1: Intra- and inter-day validation of the method for quantitative determination of curcumin and piperine

	Curcumin theoretical concentration (ng/mL)			Piperine theoretical concentration (ng/mL)		
	2.0	10.0	80.0	2.0	30.0	500.0
<i>Inter-day</i>						
Day 1						
Mean (n=5)	1.90±0.15	10.84±0.32	81.10±4.25	1.89±0.09	31.15±0.74	491.24±32.33
CV (%)	8.00	2.92	5.24	4.86	2.37	6.58
Accuracy (%)	94.90	108.36	101.37	94.60	103.82	98.25
Day 2						
Mean (n=5)	1.89±0.15	10.00±0.92	79.22±3.92	1.91±0.09	30.92±1.26	496.67±25.29
CV (%)	8.03	9.22	4.95	4.72	4.06	5.09
Accuracy (%)	94.60	99.98	99.02	95.30	103.053	99.33
Day 3						
Mean (n=5)	1.89±0.03	9.66±0.41	79.58±4.16	1.89±0.14	30.52±2.29	494.25±20.91
CV (%)	1.52	4.19	5.23	7.22	7.50	4.23
Accuracy (%)	94.40	96.6	99.48	94.60	100.41	98.85
<i>Intra-day</i>						
Mean (n=15)	1.88±0.09	10.84±0.32	86.10±5.22	1.94±0.24	29.75±1.47	493.54±33.41
CV (%)	4.74	2.92	6.06	12.36	4.94	6.77
Accuracy (%)	94.20	108.36	107.62	95.48	100.19	99.75

Short-term stability at room temperature

We evaluated short-term stability in this study where three aliquots of each test sample were left at room temperature and analysed after 12 h. This short-term stability experiment revealed no significant degradation for the two compounds in human plasma when left at room temperature over a period of 12 h, with CV of 2.77 - 6.20 % for curcumin and 2.14 - 4.85 % for piperine.

Plasma sample freeze-thaw stability

The results indicate that both curcumin and piperine were stable in plasma after three freeze-thaw cycles, with CV of 1.64 - 6.12 % for curcumin and 1.77 - 5.24 % for piperine.

Long-term stability of frozen plasma at -70 °C

Long-term stability was evaluated by analyzing the plasma test samples after storage at -70 °C for up to 2 months. The results showed that both curcumin and piperine were stable in the plasma matrix at

-70 °C for 2 months. For curcumin, the accuracy was in the range 99.30 - 100.33 % with CV between 2.27 and 7.77 %, while for piperine, accuracy and CV were in the range 97.80 - 102.99 and 1.90 - 5.11 %, respectively.

Human plasma levels

Typical chromatograms of clinical samples evaluated are shown in Fig 3. The plasma concentration profile obtained as a function of time following oral dosing of 9 healthy volunteers is shown in Fig 4.

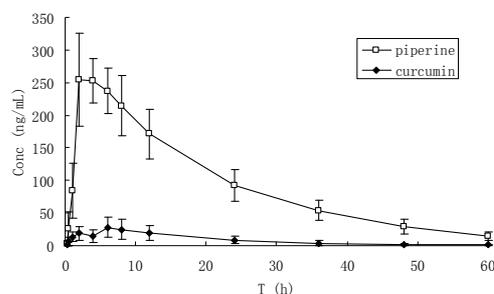


Fig 4: Mean plasma concentration versus time profiles of oral administered curcumin (—◆—) and piperine (—□—) in 9 healthy volunteers

toxicokinetic, pharmacokinetic, bioavailability, and bioequivalence studies.

DISCUSSION

A sensitive and reliable LC-MS/MS method has been developed for the determination of curcumin and piperine in human plasma. The specificity of the extraction indicate that the chromatographic method tested possessed the capacity to differentiate and quantitate the analyte in the presence of other endogenous constituents in samples, as well as to detect potential interferences. The analytical procedure used was based on liquid-liquid extraction to obtain sufficiently high recoveries. Significantly lower limit of quantitation was achieved in plasma compared to previously published methods [9,16], with LLOQ as low as 1.0 ng/ml for curcumin and 0.5 ng/ml for piperine. No interfering peaks were observed and no significant peaks were found at the retention times of the analytes using the LLE step for both analytes.

Since plasma is a complex biological matrix, any other endogenous compounds which co-elute with the analytes may cause ion suppression or enhancement, which in turn makes the quantitation of the analytes inaccurate [3,4]. In the present study, plasma matrix effect was tested by comparing chromatographic peak areas from neat standards and blank plasma extracts spiked with neat standards at concentrations of the test samples of the two compounds. The results obtained showed that the matrix effect of the plasma extract was insignificant.

CONCLUSION

The HPLC-MS/MS technique presented in this work for the simultaneous determination of curcumin and piperine in human plasma is highly selective and rugged for routine measurement of these drugs in combination therapy. The results of this study indicate that the method would be applicable to

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