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Curcumin in plasma and urine: quantitation by high-performance liquid chromatography

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Abstract

Curcumin, a derivative of the plant *Curcuma longa*, is used extensively in the food industry. It is a major component of curry powder, and research has shown that curcumin may prevent cancer and other chronic diseases. We have developed a robust automated analytical method for the determination of curcumin in plasma and urine. The method involves extracting the curcumin from 0.2 ml sample volume with ethyl acetate/methanol organic solvents, and use of an internal standard, β-17-estradiol acetate. Analysis utilizes a reversed-phase C_{18} column and UV detection at 262 nm. Performance characteristics have been assessed. The assay is linear from 0.2 to 7.0 μg/ml. The coefficient of variation for intra- and inter-day assays is <7.5%. The average recovery of curcumin from plasma and urine is greater than 96%. The data presented in this report demonstrate that the method provides rapid, sensitive, precise and accurate measurements of curcumin concentrations in plasma and urine.

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1. Introduction

In recent years, there has been growing interest in the compound, curcumin. Curcumin is a derivative of the plant *Curcuma longa*. It is widely used in the food industry as a natural food coloring agent, and it is a major component of curry powder. More recently, laboratory and clinical research has shown that curcumin has the potential to contribute to the prevention of cancer and other chronic diseases, due to various biological activities. In preclinical studies involving cell cultures, curcumin has been shown to have antiproliferative effects on human breast carcinoma cells and to induce apoptosis of the myelogenous leukemia HL-60 tumor cells [1,2]. Additionally, studies have demonstrated that dietary curcumin significantly inhibits colon tumorigenesis and tumor size in animal models [3,4]. Furthermore, curcumin has demonstrated antimutagenic effect on chromosomal aberrations in Wistar rats [5] and a

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chemopreventive effect in glandular stomach carcinogenesis [6].

More recently, Gescher et al. [7] have suggested that because curcumin has known antitumor and chemopreventive activities, the time is appropriate for phase I chemoprevention studies in persons at high risk or in patients with established malignant disease. In phase I studies, the essential requirement and goal is to establish pharmacokinetic properties in humans and pharmacological levels that are clearly associated with safety and efficacy of the drug or chemopreventive compound in a dose-related manner. Therefore, a sensitive and reliable analytical method is necessary to quantitate the compound of interest.

The initial structure of curcumin ($C_{21}H_2OO_6$) was described in 1815 by Vogel and Pellatier [8]. The structure of the curcumin was shown to be diferuloylmethane by Lampe et al. in 1910 [9]. Further work by Lampe and Milobedzska in 1913 resulted in the synthesis of the compound [10].

One of the earliest uses of chromatography to separate and quantify curcumin was described by Srinivasan in 1953 [11]. That chromatographic method involved separation of curcumin using a liquid chromatographic procedure and spectrophotometric detection. In 1978, Holder et al. [12] used a radiolabeling technique and mass spectrophotometry to quantify curcumin and metabolites of curcumin. In 1981, Ravindranath and Chandrasekhara [13] also used a radiolabeling technique and infrared spectrum to identify curcumin. Further research by Cooray [14] involved use of thin-layer chromatography combined with other techniques, including ultraviolet and densitometric detections with gas chromatography and mass spectrometry, to quantify curcuminoid content of various specimens.

Although the chemical structure of curcumin was determined in 1910 [9], it was not until the 1970s that the potential medicinal benefits of curcuminoid became the subject of scientific investigations, as described by Majeed et al. [15]. The reported medicinal benefits of curcumin highlighted the urgent need for analytical techniques to quantify curcumin as a compound under investigation for clinical effects. In response to the need for a sensitive and reliable analytical method to quantify the compound of interest, methods utilizing high-performance liquid chromatography (HPLC) techniques were described by Majeed et al. [15], Smith and Witowska [16], and Cooper et al. [17] in 1994 and 1995. However, these early quantitative methods were used to quantitate curcumin in an aqueous-based matrix.

Thus, the purpose of this study was to develop an HPLC method for quantifying curcumin in human plasma and urine.

2. Experimental methods

2.1. Chemicals

Curcumin was provided by Dr. Dean Brenner, University of Michigan, and β-17-estradiol acetate was obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, acetic acid, ethyl acetate and de-ionized water were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Instrumentation and equipment

The HPLC system consisted of a 9100 auto-sampler with refrigeration unit, a 9050 UV visible detector and a 9010 solvent delivery system, with Star 5.3 chromatography software (Varian, Walnut Creek, CA, USA). Chromatographic separation was accomplished using a Waters SymmetryShield 3.9× 150 mm, 5-μm C_{18} column (Waters, Milford, MA, USA). The column was coupled to an Alltech absorbosphere 30×4.6 mm, C_{18} guard column (Alltech Associates, Deerfield, IL, USA).

2.3. Mobile-phase reagent preparation

In a 4-l container, the following reagent volumes were measured: acetonitrile 1640 ml, methanol 920 ml, de-ionized water 1440 ml, and acetic acid 40 ml. The mixed reagent was filtered under vacuum through a 0.45-μm filter (Millipore, Bedford, MA, USA). The reagent was degassed before use with a solvent de-gas system (Fisher Scientific). This reagent is stable at room temperature for at least 4 weeks.

2.4. Extracting reagent preparation

One hundred and ninety ml ethyl acetate and 10 ml methanol were mixed together. This reagent is stored at room temperature and is stable for at least 4 weeks.

2.5. Standard and quality control sample preparation

A sample of 5.0 mg of powdered curcumin was weighed on a Mettler model AB204 balance (Mettler Instrument, Hightstown, NJ, USA). The curcumin was dissolved and made up to a volume of 25 ml in a volumetric flask with methanol to achieve a final concentration of 200 μg/ml.

Next, a working stock solution of curcumin was prepared as indicated: to 0.5 ml of curcumin stock standard (200 μg/ml), 9.5 ml of mobile phase reagent were added. This working stock standard was prepared fresh as required. Calibration was performed, using the established spiking technique of addition, to spike plasma or urine with known amounts of the stock standard.

For quality control samples, some of the prepared curcumin standards were re-assayed. Plasma samples were previously collected using heparinized vacutainer tubes. The samples were stored at −80 °C. A total of six independent plasma matrices were prepared and each plasma matrix was spiked with varying amounts of curcumin from the previously prepared stock solutions.

2.6. Internal standard preparation

Internal standard preparation was achieved by the following: 6.25 mg β-17-estradiol acetate was weighed and dissolved in 1 ml methanol with the aid of a magnetic stirrer (Corning Glassware, Corning, NY, USA). Mobile-phase reagent was added to the solution and brought to volume in a 25-ml volumetric flask. This reagent, when stored at −80 °C, is stable for at least 3 months, based on chromatographic evidence indicating no apparent deterioration during that time period. This internal standard concentration is 250 μg/ml.

2.7. Analytical procedure and sample preparation

This process begins with the following procedure: 200 μl of standard, unknown and quality control sample each were pipetted, using a micropipette (Rainin, Emeryville, CA, USA), into 2-ml micro-centrifuge tubes (USA Scientific, Ocala, FL, USA). To each tube, 80 μl de-ionized water were added. The tubes were capped and mixed for 20 s at medium speed setting by vortex (Fisher Scientific). Forty μl internal standard (250 μg/ml β-17-estradiol acetate) were added to each tube. The tubes were capped and mixed by vortex, as described above. Five hundred μl extraction reagent, 95% ethyl acetate/5% methanol, were added to each tube. This solution was capped and mixed by vortex at high-speed setting for 30 s. Next, all tubes were centrifuged at 13 500 rpm for 5 min in an Eppendorf micro-centrifuge (Brinkman Instruments, Westbury, NY, USA). After centrifugation, the upper organic layer, about 420 μl volume, was carefully removed into a clean micro-centrifuge tube. This organic layer was dried under a stream of nitrogen gas using low heat setting.

The extracted dried product was resuspended in 200 μl of prepared mobile phase reagent. The tubes were capped and mixed by vortex at medium speed for 30 s. The tubes were left at room temperature in the dark for at least 10 min, followed by a repeat vortex mixing and transfer of the content of each tube to an injection sample vial (about 180 μl) for HPLC assay.

2.8. HPLC analytical run

Curcumin in plasma and urine was separated and quantified by isocratic HPLC method using ultraviolet (UV) detection at a wavelength of 262 nm. An aliquot (20 μl) was injected onto a reversed-phase column and eluted with a mobile phase containing a mixture of acetonitrile–methanol–water–acetic acid (41:23:36:1, v/v/v/v). Flow rate was 1.0 ml/min. The quantitation of curcumin is by peak area ratio (curcumin to internal standard) and is based on a standard curve in a plasma or urine matrix, generated by using an external standard to spike plasma or urine. A linear curve is generated from a single analysis of six different standard concentrations.

Table 1
Summary of results for recovery of added curcumin

Sample type	Calculated concentration (μg/ml)	Number of assays	Recovered concentration (Mean [SD]) (μg/ml)	Coefficient of variation (%)	Recovery (%)
Plasma, low	0.250	5	0.243 (0.006)	2.20	97.2
Plasma, medium	1.000	5	1.016 (0.010)	1.00	98.4
Plasma, high	4.000	5	4.018 (0.010)	0.25	99.6
Urine, low	0.125	5	0.113 (0.006)	5.00	98.8
Urine, medium	0.500	5	0.479 (0.010)	1.99	95.9
Urine, high	3.000	5	3.369 (0.062)	1.85	112

3. Validation results

3.1. Recovery of added curcumin

The amount of added curcumin recovered from plasma and urine pool samples was estimated in the high, medium and low concentration ranges. These concentrations were 0.250, 1.000 and 4.000 μg/ml, respectively, for plasma, and 0.125, 0.500 and 3.000 μg/ml, respectively, for urine. These sample pools were prepared by the standard addition technique. Five estimations were made on each sample pool. The results are summarized in Table 1 and indicate a recovery of added curcumin >96% in the plasma and urine matrices.

3.2. Extraction efficiency

To determine the extraction efficiency of the method, the neat solution of curcumin containing the internal standard was analyzed at the low (0.2 μg/ml) and high (5.0 μg/ml) levels of concentration. Additionally, the corresponding low and high levels of curcumin in urine and plasma matrices were extracted with internal standard. All samples were assayed in the same analytical run.

The results are summarized in Table 2. The results show the extraction efficiency of plasma and urine at the low concentration level was 88.0 and 70.0%, respectively. At the high concentration level, the extraction efficiency was 97.0% for both plasma and urine. The extraction efficiency for the internal standard was between 95 and 99.5%, as shown in Table 3.

3.3. Estimation of accuracy and precision

Accuracy and precision was assessed from the results of replicate assays on three different sample pools in the high, medium and low concentration range. These samples were prepared in plasma and urine matrices, by the standard addition technique. Five estimations were made on each sample pool during a 4-day period. The pool samples were stored at −80 °C as single aliquots throughout the period of the experiment. The results are summarized in Table

Table 2
Summary of extraction efficiency results

Sample type	Concentration (μg/ml)	Number of assays	Area count (Mean [SD])	Coefficient of variation (%)	Extraction efficiency (%)
Curcumin, neat	0.2	5	4915 (29)	0.6	100
Extracted urine	0.2	5	3452 (197)	5.7	70
Extracted plasma	0.2	5	4331 (175)	4.0	88
Curcumin, neat	5.0	5	126 712 (287)	0.2	100
Extracted urine	5.0	5	122 715 (3070)	2.5	97
Extracted plasma	5.0	5	122 654 (6020)	4.9	97

Table 3
Summary of extraction efficiency results: internal standard

Sample	Concentration (μg/ml)	Number of assays	Area count (Mean [SD])	Coefficient of variation (%)	Extraction efficiency (%)
Internal standard, neat	250.00	5	76 133 (1117)	1.5	100.0
Extracted urine low	250.00	5	75 734 (152)	0.2	99.5
Extracted urine high	250.00	5	72 379 (1309)	1.8	95.0
Extracted plasma low	250.00	5	73 987 (857)	1.1	97.0
Extracted plasma high	250.00	5	72 228 (3983)	5.5	94.9

4. The mean values (accuracy) of the prepared samples in plasma and urine matrices were within 96% of their actual concentrations.

3.4. Precision around the standard curve

The precision around the standard curve was calculated as a ratio of the area count of the curcumin divided by the area count of the internal standard. For the plasma standard curve, six different concentration points were prepared, covering the range 0.2–7.0 μg/ml. Each standard was analyzed once during a 4-day period. The precision was determined to be between 6.2 and 12.7% (see Table 5).

For the urine standard curve, seven different concentration points were prepared covering the range 0.2–7.0 μg/ml. Each standard was analyzed once during a 4-day period. The precision was determined to be between 2.3 and 12.2%.

Table 4
Summary of within-run and run-to-run variation results

Sample type	Calculated concentration (μg/ml)	Number of assays	Concentration (Mean [SD]) (μg/ml)	Coefficient of variation (%)	Day of analysis
Plasma, low	0.125	5	0.123 (0.001)	0.8	1
	0.125	5	0.126 (0.003)	2.2	2
	0.125	5	0.126 (0.008)	6.2	3
	0.125	5	0.126 (0.004)	3.4	4
Plasma, medium	0.500	5	0.501 (0.010)	1.9	1
	0.500	5	0.501 (0.004)	0.8	2
	0.500	5	0.510 (0.005)	0.4	3
	0.500	5	0.521 (0.006)	1.1	4
Plasma, high	3.000	5	3.051 (0.025)	0.8	1
	3.000	5	2.734 (0.194)	7.1	2
	3.000	5	2.953 (0.058)	2.0	3
	3.000	5	3.063 (0.029)	1.0	4
Urine, low	0.200	5	0.214 (0.003)	1.5	1
	0.200	5	0.211 (0.004)	1.9	2
	0.200	5	0.205 (0.004)	2	3
	0.200	5	0.202 (0.003)	1.6	4
Urine, medium	1.000	5	0.997 (0.038)	3.8	1
	1.000	5	1.060 (0.024)	2.2	2
	1.000	5	1.049 (0.023)	2.2	3
	1.000	5	1.039 (0.022)	2.1	4
Urine, high	4.000	5	3.867 (0.096)	2.5	1
	4.000	5	4.090 (0.064)	1.6	2
	4.000	5	4.209 (0.160)	3.8	3
	4.000	5	4.080 (0.023)	0.6	4

Table 5
Summary of standard precision in plasma and urine as a ratio of curcumin to internal standard

Sample type	Number of days	Ratio of curcumin/internal standard area count (Mean [SD])	Coefficient of variation (%)
Plasma			
0.2 μg/ml	4	0.060 (0.006)	10.0
0.4 μg/ml	4	0.110 (0.014)	12.7
0.8 μg/ml	4	0.216 (0.026)	12.0
1.6 μg/ml	4	0.500 (0.050)	10.0
3.2 μg/ml	4	1.020 (0.114)	11.2
7.0 μg/ml	4	1.920 (0.121)	6.2
Urine			
0.2 μg/ml	4	0.039 (0.001)	3.8
0.4 μg/ml	4	0.098 (0.004)	4.3
0.8 μg/ml	4	0.210 (0.006)	2.7
1.6 μg/ml	4	0.473 (0.011)	2.3
3.2 μg/ml	4	1.045 (0.044)	4.2
5.0 μg/ml	4	1.657 (0.107)	6.4
7.0 μg/ml	4	2.287 (0.280)	12.2

3.5. Assessment of stability under freeze/thaw conditions

Stability of the curcumin in plasma and urine matrices was assessed from the results of replicate assays on three different sample pools. These sample pools were prepared by the standard addition technique. No preservative was added to any of the pool samples.

Five estimations were made on each pool during three freeze–thaw cycles. Each pool sample was assayed on day number one, and subsequently, the balance of each pool sample was frozen at −80 °C. On each succeeding day (cycle), the sample pool was removed from the freezer allowed to thaw at room temperature in the dark. After thawing, the samples were mixed well by gently vortexing. Next, an aliquot of 200 μl was removed and assayed. The remainder of the pool samples were re-frozen at −80 °C. This procedure was repeated for three freeze–thaw cycles.

The results are summarized in Table 6. The mean value of each sample pool in plasma and urine matrices after three freeze–thaw cycles were within 91% of their original prepared baseline values.

3.6. Assessment of the limit of detection

The limit of detection was determined using four different sample pools. These were prepared using standard addition technique in plasma and urine matrices. The concentration of each pool was calculated to reflect values below the first accepted point on the established standard curve (0.2 μg/ml). Five independent assays were done on each sample pool, and the results are summarized in Table 7.

The limit of detection, the lowest concentration on the curcumin standard curve that was measured with acceptable accuracy, precision and variability, was determined to be 0.063 μg/ml in plasma. This value was within 5% of the original calculated plasma concentration. The limit of detection obtained for the urine matrix was 0.091 μg/ml. This value was within 1% of the original calculated urine concentration.

4. Discussion

Several HPLC methods to measure curcumin have been described. However, these early methods of quantitation were used in aqueous-based matrix only. Thus, the major disadvantage of these early methods was the absence of a protein extraction methodology suitable for use with plasma and urine. The HPLC method of Ireson et al. [18] shows promise for meeting the analytical requirements of clinical research. The disadvantages of this method are a long analytical run time of 50 min and the use of an internal standard, tetra-(*m*-hydroxylphenyl)-chlorine, that is not easily obtainable and requires a double extraction step. Furthermore, the previously reported HPLC method of Kurano and Ho [19] used an internal standard, *O*-nitroaniline, for aqueous extractions of curcuminoid compounds; however, *O*-nitroaniline is a very toxic compound which is implicated in methemoglobinemia, cyanosis and liver damage. This internal standard is thus not recommended for routine use. With the rapidly expanding research involving the use of curcumin and the clinical effects of curcumin, it is therefore necessary to have a reliable method of extraction of curcumin in blood plasma and urine.

The need is immediate, as phase I clinical trials

Table 6
Summary of plasma and urine freeze–thaw cycle results

Sample type	Number of assays	Freeze–thaw cycle number	Baseline concentration (μg/ml)	Concentration (Mean [SD]) (μg/ml)	Coefficient of variation (%)
Plasma					
Pool 1	5	0	0.125	0.119 (0.008)	6.7
	5	1	0.125	0.123 (0.006)	4.6
	5	2	0.125	0.124 (0.003)	2.7
	5	3	0.125	0.122 (0.005)	3.9
Pool 2	5	0	0.500	0.492 (0.005)	1.0
	5	1	0.500	0.472 (0.010)	2.2
	5	2	0.500	0.499 (0.016)	3.2
	5	3	0.500	0.493 (0.008)	1.7
Pool 3	5	0	2.000	2.035 (0.025)	1.3
	5	1	2.000	2.186 (0.007)	3.1
	5	2	2.000	1.848 (0.038)	2.0
	5	3	2.000	2.017 (0.063)	3.1
Urine					
Pool 1	5	0	0.400	0.414 (0.011)	2.6
	5	1	0.400	0.440 (0.023)	5.3
	5	2	0.400	0.408 (0.009)	2.3
	5	3	0.400	0.405 (0.019)	4.8
Pool 2	5	0	1.500	1.526 (0.041)	2.7
	5	1	1.500	1.511 (0.032)	2.1
	5	2	1.500	1.423 (0.012)	0.8
	5	3	1.500	1.391 (0.047)	3.3
Pool 3	5	0	3.000	3.097 (0.089)	2.9
	5	1	3.000	3.030 (0.065)	2.1
	5	2	3.000	2.827 (0.053)	1.9
	5	3	3.000	2.74 (0.047)	1.7

Table 7
Summary of limit of detection results for plasma and urine

Sample type	Number of assays	Baseline concentration (μg/ml)	Mean (SD) (μg/ml)	Coefficient of variation (%)
Plasma				
Pool 1	5	0.060	0.063 (0.005)	7.7
Pool 2	5	0.070	0.070 (0.002)	2.3
Pool 3	5	0.080	0.093 (0.007)	7.6
Pool 4	5	0.090	0.094 (0.004)	4.2
Pool 5	5	0.100	0.105 (0.006)	6.1
Pool 6	5	0.125	0.136 (0.003)	2.5
Urine				
Pool 1	5	0.090	0.091 (0.006)	6.5
Pool 2	5	0.100	0.101 (0.009)	8.8
Pool 3	5	0.125	0.127 (0.002)	1.6
Pool 4	5	0.200	0.203 (0.008)	0.4

involving the use of curcumin have already begun. Cheng et al. [20] recently reported the results of a clinical toxicity study. In that study, they reported that the maximum tolerable oral dose of curcumin was 8000 mg/day, due to the bulky volume of the curcumin that was reported to be unacceptable to the patients. They did not identify curcumin in the urine of the study participants. However, low bioavailability of the formulation used may be the reason for the lack of detection of curcumin in urine, because it has been reported that curcumin has a low absorption rate in the gut [21].

The analytical method used for curcumin in the study by Cheng et al. [20], the HPLC method of Cooper et al. [17], was originally developed for the analytical assay of curcumin in an aqueous matrix, without the addition of an internal standard. The extraction efficiency for curcumin has not been described for this method. Therefore, it is possible that method sensitivity may be a factor in the non-detection of curcumin in the urine of patients from the reported phase I study of Cheng et al. [20].

Other clinical studies of the pharmacokinetic properties of curcumin are currently underway. Fig. 1 shows the results of extraction of an ingested dose of curcumin from human plasma. The curcumin was ingested in a capsular form at a dose level of 12 000 mg, and blood utilized in this assay was obtained 2 h after ingestion of the curcumin.

The use of an internal standard in extraction techniques and method is essential to compensate for extraction variation, efficiency and analytical errors. Thus, recognizing the important role of the internal standard in the analytical process, the method we developed includes an internal standard, β-17-estradiol acetate. This compound, β-17-estradiol acetate, is not structurally similar to the analyte of interest, curcumin. However, its use as an internal standard is scientifically acceptable, because the behavioral characteristics and properties of β-17-estradiol acetate conform to the chemical requirement for internal standards in HPLC, according to Snyder et al. [22]. More specifically, β-17-estradiol acetate is well-resolved from the compound of interest and other peaks. It is not found in the original sample sources (plasma and urine), and its characteristics mimic the analyte during sample preparation steps [22]. Additionally, β-17-estradiol acetate is commercially available in high purity, it is stable and nonreactive with sample or mobile phase, and it has a similar detector response to the analyte for the concentration used [22].

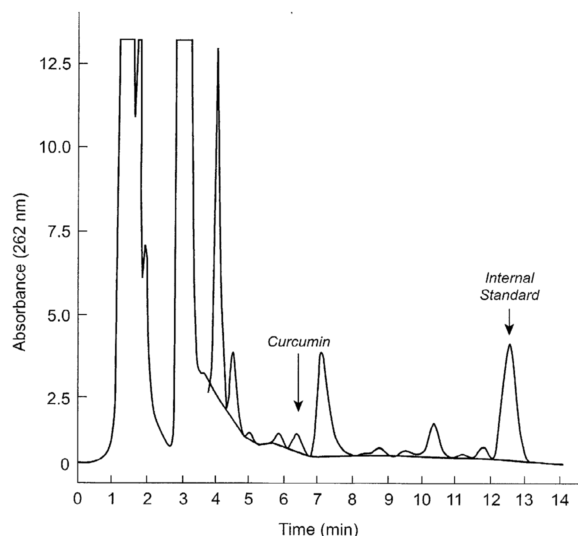

Fig. 1. High-performance liquid chromatogram of plasma. Curcumin was extracted from blood collected 2 h post-ingestion of 12 000 mg of curcumin complex in capsular form. Waters SymmetryShield C_{18} column (150×3.9 mm, 5 μm particle size), ultraviolet detection at 262 nm, flow-rate 1.0 ml/min, and mobile phase of acetonitrile–methanol–water–acetic acid (41:23:36:1, v/v/v/v).

5. Conclusion

We have developed a simple HPLC method for the analysis of curcumin in plasma and urine. The method presented here can be used as an analytical tool for the determination of plasma and urine curcumin levels in clinical phase I trials. This method has the advantage of being relatively simple and is practical for ease of use in clinical applications. The method is rapid, accurate, precise, easily standardized and capable of handling large batches with short analysis time.

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