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Sensitive and rapid isocratic liquid chromatography method for the quantitation of curcumin in plasma

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Abstract

An HPLC assay was developed using three methods of plasma sample preparation in order to quantitate curcumin, the main constituent in the herbal dietary supplement turmeric. Each method involves simple and rapid processing of samples (either an ethyl acetate or chloroform extraction) with resulting different quantitation limits for curcumin. The assay was developed in an effort to quantify extremely low curcumin plasma concentrations observed in preliminary in vivo studies. The most sensitive assay can reliably detect concentrations down to 2.5 ng/ml. Plasma quantitation was precise and accurate based on both intra- and inter-day validations as indicated by low values for coefficients of variation and bias, respectively ($\leq$15%). The analytical validation was reproducible between different analysts. The resulting analytical method couples desired sensitivity with the ease of an isocratic system.

Keywords: Curcumin

1. Introduction

Turmeric, an herb originating from the rhizome of the plant *Curcuma longa*, is most widely known for its use as a spice in cooking and as a coloring agent for foods, such as mustard and pickles. Interest in this herb has grown in recent years based on its putative beneficial pharmacological effects including antioxidant [1,2], anti-inflammatory [3,4], and cancer chemopreventive actions [5–7]. Curcumin, the major yellow-orange pigment extracted from turmeric, may be responsible for much of the bioactive effects. In a recent study, products of curcumin reduction and conjugation had a reduced ability to inhibit cyclooxygenase-2 (COX-2) expression, which correlated to a decrease in the inhibition of prostaglandin biosynthesis when compared to intact curcumin, indicating that the metabolic conversion of curcumin results in pharmacologic deactivation [8]. Curcumin is also a potent scavenger of various reactive oxygen species (ROS) including superoxide anions [2] and hydroxyl radicals [2,9]. In addition, there have been indications that curcumin may help prevent and treat patients with Alzheimer's disease by reducing oxidative damage, plaque burden, and suppressing specific inflammatory factors [10].

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Surprisingly, several studies have reported poor bioavailability of curcumin in both rodents and humans despite the promising biological effects that have been observed. The suspected cause may be poor absorption due to its extremely low aqueous solubility and/or extensive pre-systemic metabolism. In a study by Wahlstrom and Blennow [11], curcumin was undetectable in plasma 1 h following a single oral dose of 1 g/kg given to rats. The resulting plasma concentrations were calculated to be no greater than 20 ng/ml. Similarly, in another study, a 1 g/kg oral dose administered to mice resulted in a maximum plasma concentration of 220 ng/ml. Other samples had lower concentrations including concentrations below the quantifiable limit of 73.7 ng/ml [12]. A recent study by Ireson et al. [8] also reported that plasma concentrations were below the limit of quantitation (7 ng/ml) after the administration of a 500 mg/kg oral dose to rats. An investigation in humans found no detectable concentrations of curcumin following a 180 mg oral dose [13]. Even doses as high as 8 g curcumin per day administered to seriously ill human subjects result in only an average peak serum concentration of 652.5 ng/ml [14].

A limitation to the studies cited above was the inability to quantitate low curcumin concentrations. Quantitation of curcumin concentrations below 10 ng/ml would allow better characterization and understanding of the disposition and absorption kinetics of this compound. Although several methods of detection for curcumin have been published, only one has reported a limit of quantitation below 10 ng/ml [8]. Of these methods, several involve spectrophotometric [15], liquid chromatography-mass spectrophotometric [16,17], and radiolabeled determination of curcumin [18]. HPLC methods have also been developed in order to quantitate curcumin in biological samples [8,12,19,20]. Ireson et al. [8] utilized a HPLC gradient system that produced reasonable separation and sensitivity (limit of quantitation: 7.37 ng/ml; limit of detection: between 1.84 and 3.68 ng/ml). The retention time for curcumin, however, was greater than 35 min. Investigators using isocratic HPLC systems had the convenience of shorter retention times (ranging from 7 to 25 min) [12,19,20], but lacked the sensitivity reported when using a gradient method (73.7 ng/ml for isocratic versus 7.37 ng/ml for gradient) [8,12].

In investigations in this laboratory into the disposition kinetics, bioavailability, and anti-inflammatory effects of curcumin, our goal was to develop a simple liquid–liquid extraction and assay that combines the sensitivity attained from a gradient method with a short retention time achieved using an isocratic method. During the development and refining of the analytical assay, an isocratic HPLC assay using different sample preparation protocols and with varying limits of quantitation were validated from plasma.

2. Experimental

2.1. Chemicals

Curcumin (Fig. 1) was isolated and purified from the turmeric rhizome by Dr. Shivanand Jolad (University of Arizona). Purity (>97%) was determined using HPLC, NMR, elemental analysis, and melting point. β-Estradiol, sodium phosphate dibasic (heptahydrate), and potassium hydroxide were purchased from Sigma (St. Louis, MO, USA). Citric acid (anhydrous) and HPLC-grade methanol, ethyl acetate, chloroform, and tetrahydrofuran were purchased from VWR Scientific (South Plainfield, NJ, USA).

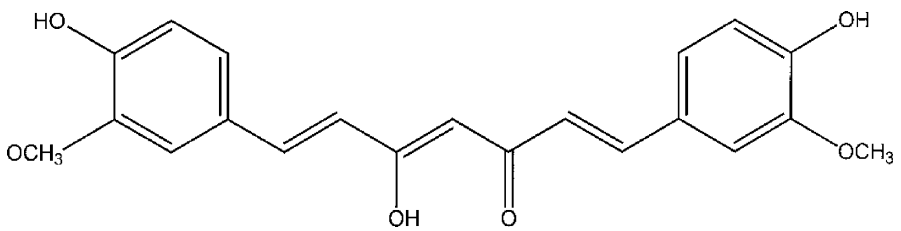

Fig. 1. Chemical structure of curcumin.

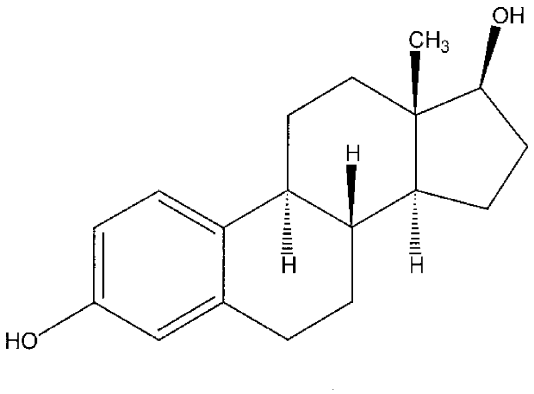

Fig. 2. Chemical structure of the internal standard, β-estradiol.

2.2. Equipment

The HPLC system included a Waters 717-plus autosampler with a refrigeration unit, a Waters 996 photodiode array detector, and a Waters 515 pump (Milford, MA, USA). The Waters Millennium[32] chromatographic software (version 3.20) was utilized for integration (Milford, MA, USA). A visible wavelength of 430 nm was used to detect curcumin. To detect the internal standard β-estradiol (Fig. 2), an ultraviolet wavelength of 280 nm was used.

2.3. Sample preparation

Blood was obtained from male uncastrated Yucatan micropigs (S & S Farms, Rachita, CA, USA) via a vascular access port (Access Technologies, Skokie, IL, USA) and was transferred to 10 ml Vacutainer tubes containing sodium heparin (VWR Scientific, South Plainfield, NJ, USA) to prevent coagulation. After gentle inversion of tubes, blood was centrifuged at 2000 × *g* for 10 min to separate red blood cells from plasma. Plasma was transferred to clean 15 ml polypropylene screw cap tubes and stored in a −70 °C freezer until use.

2.4. Liquid–liquid extraction methods

2.4.1. Ethyl acetate extraction: linear range (25–250 ng/ml)

One milliliter blank pig plasma in 12 mm × 75 mm borosilicate glass test tubes was spiked with 20 μl of methanolic standards (1.25–12.5 μg/ml) to produce plasma curcumin concentrations ranging from 25 to 250 ng/ml. One-hundred microliter 0.1 M citrate-phosphate buffer (pH 3.0) was added to each sample. Two milliliter of ethyl acetate was used to extract curcumin. Samples were shaken on a horizontal shaker set on "high" for 10 min followed by centrifugation at 1200 × *g* for 10 min. A fixed volume of ethyl acetate (1.65 ml) was transferred to a new glass test tube and evaporated to dryness using a Speed-Vac (Thermo Savant, Holbrook, NY, USA) for approximately 75 min. Samples were reconstituted in 100 μl methanol, and 50 μl was injected onto the HPLC.

2.4.2. Chloroform extraction: linear range (5–25 ng/ml)

Two milliliter blank pig plasma in 12 mm × 75 mm borosilicate glass test tubes was spiked with 40 μl of methanolic standards (0.25–1.25 μg/ml) to produce plasma curcumin concentrations ranging from 5 to 25 ng/ml. Three-hundred microliter 0.1 M citrate-phosphate buffer (pH 3.0) was added to each sample. Two milliliter chloroform was used to extract curcumin. Samples were shaken on a horizontal shaker set on "high" for 10 min followed by centrifugation at 1200 × *g* for 10 min. A fixed volume of chloroform (1.7 ml) was transferred to a new glass test tube and evaporated to dryness using a Speed-Vac for approximately 30 min. Samples were reconstituted in 75 μl methanol, and 50 μl was injected onto the HPLC.

2.4.3. Chloroform extraction: linear range (2.5–50 ng/ml)

Two milliliter blank pig plasma in 12 mm × 75 mm borosilicate glass test tubes was spiked with 20 μl methanolic standards (0.25–5 μg/ml) to produce plasma curcumin concentrations ranging from 2.5 to 50 ng/ml. Twenty microliter of the internal standard, β-estradiol in methanol (20 μg/ml), was added to each sample to give a final plasma concentration of 200 ng/ml. Two milliliter chloroform was used to extract both curcumin and β-estradiol. Samples were shaken on a horizontal shaker set on "high" for 10 min followed by centrifugation at 1200 × *g* for 10 min. The organic layer was transferred to a fresh glass test tube and evaporated to dryness using a Speed-Vac for approximately 30 min. Samples were reconstituted in 75 μl methanol, and 50 μl was injected onto the HPLC.

2.5. Column liquid chromatography

Separation of curcumin and β-estradiol was achieved by using an isocratic HPLC assay. Samples were injected onto a Waters SymmetryShield reversed-phase C_{18} column, 150 mm × 3.9 mm, 5 μm particle size (Milford, MA, USA), attached to a Phenomenex SecurityGuard C_{18} guard cartridge system, 4.0 mm × 3.0 mm (Torrance, CA, USA). The column was operated at ambient temperature.

2.6. Mobile phase

The mobile phase consisted of a 1% (w/v) citric acid solution, adjusted to pH 3.0 using a 45% (w/v) potassium hydroxide solution, and tetrahydrofuran in the ratio of 50:50 (v:v). The solution was filtered under a vacuum through a 0.2 μm filter (Alltech, Deerfield, IL, USA). At room temperature, the mobile phase was stable for at least 1 month. Flow rate of the mobile phase was 1.0 ml/min.

3. Results

3.1. HPLC analysis

A linear relationship between curcumin plasma concentration and response was found for the three different concentration ranges examined. In each standard curve, at least five different concentrations were used to establish the linear range. A blank sample was also assayed in order to identify any non-specific peaks that might interfere with the curcumin and/or β-estradiol peak. Coefficients of determination (r^2) ranged from 0.9978 to 0.9998 for the ethyl acetate and chloroform extraction (with internal standard) procedures. Chloroform extraction in the absence of an internal standard yielded an r^2 value no lower than 0.9873 (Table 1). HPLC analysis of curcumin revealed one peak that eluted between 4 and 6 min at a visible wavelength of 430 nm (Fig. 3a). At an ultraviolet wavelength of 280 nm, β-estradiol was detected with a retention time of about 4 min (Fig. 3b). The blank sample indicated the presence of a minor peak with the same retention times as curcumin and β-estradiol. This, however, did not hinder the reproducibility of any of the assays. These minor peaks may be due to the extraction of endogenous constituents from the pig plasma.

3.2. Accuracy and precision

Low, medium, and high concentrations were assayed in triplicate across three different days to determine the accuracy and precision of each of the three methods. The intra-day values for coefficient of variation (accuracy) and bias (precision), along with the mean, were calculated and are summarized in Table 2. For the ethyl acetate extraction, the concentrations analyzed were 25, 100, and 250 ng/ml. The concentrations examined using the chloroform extraction with and without an internal standard were 2.5, 10, 50 and 5, 10, 25 ng/ml, respectively. With the exception of the chloroform extraction in the absence of an internal standard, the coefficient of variation (CV) and bias

Table 1
Characteristics and reproducibility of the three curcumin plasma extraction methods

Extraction method	Concentration range (ng/ml)	Day of assay	Slope	Intercept	Coefficient of determination (r^2)	Recovery (%)
Ethyl acetate	25–250	1	2859	3378.2	0.9995	66.0
		2	3025	4429.9	0.9998	
		3	3013	3727.7	0.9996	
Chloroform without internal standard	5–25	1	8222	11988.0	0.9980	76.9
		2	9961	26098.0	0.9873	
		3	8762	15651.0	0.9937	
Chloroform without internal standard	2.5–50	1	0.109	0.0108	0.9995	78.0
		2	0.052	0.0068	0.9996	
		3	0.117	0.1761	0.9978	

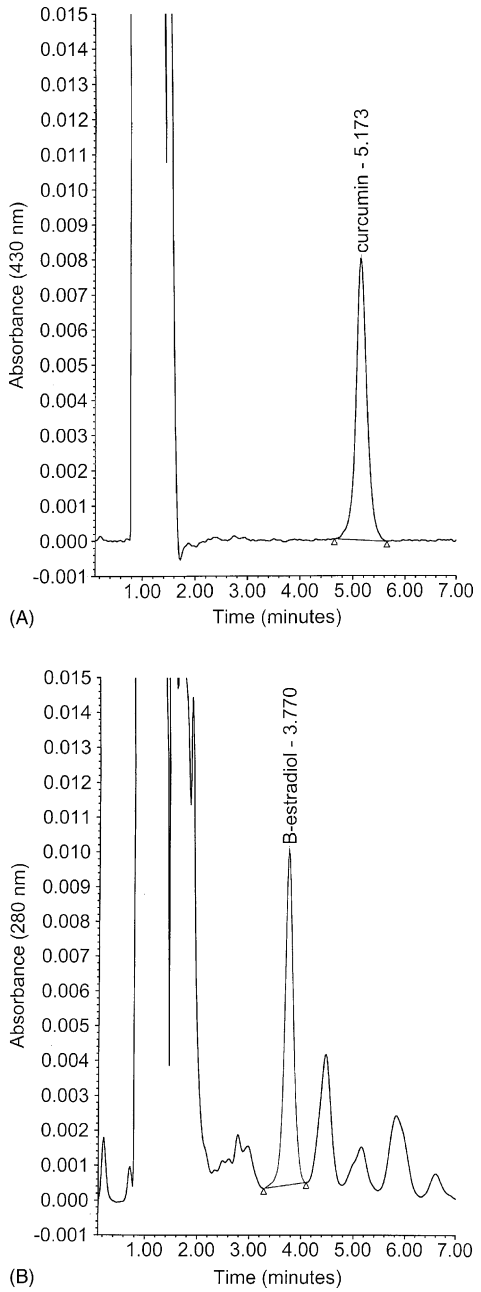

Fig. 3. (a) HPLC chromatogram obtained from extraction of pig plasma spiked with pure curcumin (10 ng/ml). Chloroform was used to extract curcumin (20 ng) from 2 ml pig plasma. Curcumin retention time is 5.173 min. Refer to text for HPLC conditions. (b) HPLC chromatogram obtained from extraction of pig plasma spiked with the internal standard, β-estradiol (200 ng/ml). Chloroform was used to extract β-estradiol from 2 ml pig plasma. Refer to text for HPLC conditions.

were no greater than 10% for any of the methods. The values for coefficient of variation and bias were lowest for the ethyl acetate extraction (CV $\leq$ 5% and bias $\leq$ 6.1%).

Inter-day values reflected much of the same results as those observed with the intra-day comparison (Table 3). Coefficients of variation and bias values were less than 12.3 and 8%, respectively, for all three methods. Again, the ethyl acetate extraction possessed the lowest values for accuracy and precision (CV and bias $\leq$ 1%).

3.3. Overall recovery

To determine the overall recovery of curcumin in each method, direct injections of the methanolic standards including the internal standard were made. These direct injection samples contained the equivalent amount of curcumin and internal standard as spiked in the extracted plasma standards. The ratios of the area of the extracted standards and the area of the direct injection samples were taken, and these values (in percent) are summarized in Table 1. The overall recovery was greatest for the chloroform extraction with the addition of the internal standard, 78%.

3.4. Reproducibility of assay

Another analyst in our laboratory was able to reproduce the values shown in Table 2. Differences between analysts did not exceed 10%. For the ethyl acetate extraction, coefficients of variation and bias values were no greater than 5 and 10%, respectively, for all concentrations. Values obtained from the chloroform extraction with the addition of β-estradiol for the low, medium, and high concentration replicates were between 1.7 and 10.8% for coefficient of variation and 0.2 and 16.9% for bias.

4. Discussion

Initially, only one of the three extraction methods, the ethyl acetate extraction, was validated during our preliminary investigation into the bioavailability of curcumin following dosing to Yucatan micropigs. After performing several preliminary bioavailability experiments, most of the plasma samples assayed

Table 2
Summary of the intra-day reproducibility of the three curcumin plasma extraction methods determined over 3 days

Extraction	Concentration (ng/ml)	Day of assay	Mean ± standard deviation (ng/ml)	Coefficient of variation (%)	Bias (%)
Ethyl acetate	25	1	26.5 ± 0.2	0.9	6.1
	25	2	24.8 ± 0.2	1.0	0.6
	25	3	23.9 ± 0.5	2.0	4.6
	100	1	102.9 ± 4.4	4.3	2.9
	100	2	94.9 ± 1.7	1.8	5.1
	100	3	99.1 ± 1.6	1.6	0.9
	250	1	253.5 ± 1.7	0.7	1.4
	250	2	245.2 ± 0.7	0.3	1.9
	250	3	254.1 ± 8.6	3.4	1.6
Chloroform without internal standard	5	1	5.1 ± 0.7	13.1	1.9
	5	2	4.5 ± 0.3	7.5	9.8
	5	3	4.3 ± 0.4	9.5	15.2
	10	1	9.2 ± 0.5	5.0	7.7
	10	2	8.7 ± 0.6	7.1	13.3
	10	3	10.4 ± 0.04	0.3	3.9
	25	1	23.3 ± 0.1	0.2	6.6
	25	2	22.4 ± 0.5	2.4	10.5
	25	3	26.9 ± 1.0	3.8	7.4
Chloroform with internal standard	2.5	1	2.6 ± 0.2	7.3	4.3
	2.5	2	2.7 ± 0.3	10.0	8.8
	2.5	3	2.3 ± 0.03	1.4	9.7
	10	1	9.4 ± 0.6	6.9	6.3
	10	2	9.1 ± 0.3	3.3	9.0
	10	3	9.7 ± 0.7	7.0	3.1
	50	1	46.0 ± 2.1	4.6	8.0
	50	2	50.6 ± 0.9	1.8	1.2
	50	3	48.8 ± 2.0	4.0	2.4

from these studies had concentrations that were below the limit of quantitation of 25 ng/ml. Although this observation supported those seen in both rodents and humans, another more sensitive assay was needed to reliably quantitate anticipated plasma concentrations in future in vivo studies. The development of the two other chloroform assays was completed, and a limit of quantitation as low as 2.5 ng/ml was achieved. A chromatogram of an authentic Yucatan micropig plasma sample obtained 15 min after the intravenous admin-

Table 3
Summary of inter-day reproducibility of the three curcumin plasma extraction methods determined over 3 days

Extraction	Concentration (ng/ml)	Mean ± standard deviation (ng/ml)	Coefficient of variation (%)	Bias (%)
Ethyl acetate	25	25.1 ± 1.2	0.1	0.3
	100	99.0 ± 4.3	0.04	1.0
	250	250.9 ± 6.2	0.02	0.4
Chloroform without internal standard	5	4.6 ± 0.6	12.3	7.7
	10	9.5 ± 0.9	9.4	5.4
	25	24.2 ± 2.1	8.8	3.2
Chloroform with internal standard	2.5	2.5 ± 0.3	10.5	1.2
	10	9.4 ± 0.6	5.9	6.1
	50	48.5 ± 2.5	5.2	3.1

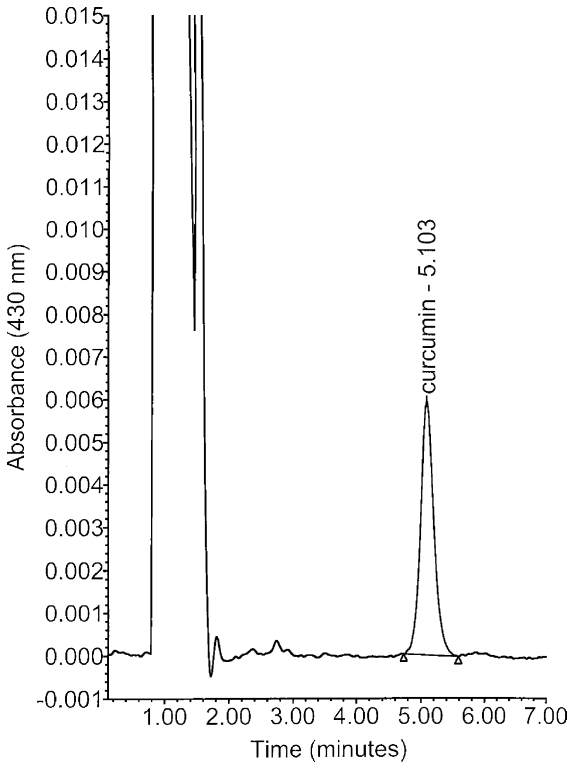

Fig. 4. HPLC chromatogram of an extracted authentic pig plasma sample obtained 15 min following an intravenous dose (8.3 mg) of curcumin. Chloroform was used to extract curcumin from 2 ml of pig plasma. Curcumin plasma concentration was determined to be 6.7 ng/ml. Internal standard was absent. Refer to text for HPLC conditions.

istration of an 8 mg dose of curcumin is shown in Fig. 4. This sample was extracted using chloroform (in the absence of an internal standard) and corresponds to a plasma concentration of 6.7 ng/ml. Commercial turmeric capsules (VitaminWorld, Ronkonkoma, NY, USA; eight capsules containing 29.6 mg curcumin) were also administered to Yucatan micropigs, which yielded concentrations that were either near or below the limit of quantitation of 2.5 ng/ml (Pak et al., unpublished observation). Although the ethyl acetate assay was not sensitive enough for our purpose, we present here the results of all three methods in order to provide investigators the option of choosing the limit of quantitation needed for their particular application.

Although there are several HPLC methods available, only the method of Ireson et al. [8] had sensitivity comparable to that obtained in our assay. The major disadvantage of the method of Ireson et al. is the lengthy retention time of curcumin (ca. 40 min), which was due to their intent of separating chromatogram peaks of curcumin and its metabolites. An isocratic method provides a shorter retention time for curcumin, as reported in several papers [12,19,20]. However, sensitivity is compromised; the lowest limit of quantitation for a previously reported isocratic method is about 70 ng/ml [12].

In terms of sample processing, the ethyl acetate extraction possesses an easier transfer step than either of the chloroform extractions because the ethyl acetate resides above the plasma sample after centrifugation while chloroform resides beneath the aqueous phase. However, of the two extraction solvents, chloroform provided the greatest recovery of curcumin from plasma and the lowest limit of quantitation. An improvement in the reliability of the chloroform extraction was the addition of an internal standard. Heath et al. [20] provide a useful internal standard, β-17-estradiol acetate, which we adapted for our assay. The internal standard was particularly helpful, especially when compensating for loss of curcumin during extraction when chloroform was transferred between tubes. In the extraction methods where internal standard was absent, any curcumin that was not transferred (when the fixed volume of the organic layer was removed) contributed to the loss in overall recovery in addition to the lack of multiple extractions, the extracting ability of ethyl acetate and chloroform, and the loss of curcumin during evaporation. Another minor alteration to this extraction was the exclusion of the 0.1 M citrate-phosphate buffer (pH 3.0). In a study by Wang et al. [21], it was reported that the stability of curcumin improved in aqueous buffer solutions between pH 3.0 and 6.5. Investigators, however, also suggest that physiological matrices containing proteins may prolong stability, from which one may infer that protein binding plays a role in protecting curcumin [12,21]. After performing our own preliminary experiments into the stability of curcumin in plasma, it was determined that curcumin was stable in plasma for at least 4 h without controlling pH (Pak et al., unpublished observation). Therefore, the addition of the acidic buffer was omitted in the chloroform extraction with the inclusion of the internal standard. It should be noted, however, that curcumin

is very unstable in whole blood (Pak et al., unpublished observation). Effort must be made to separate curcumin from blood as soon after blood sampling as possible.

An observation that was noted during the validation of the chloroform extraction with internal standard was the consistent presence of a single deviant replicate for the low standard concentration (2.5 ng/ml). The atypical replicate possessed a greater peak area than the other replicates. As a result, an extra replicate was added at this concentration. We caution investigators when using this extraction method that one may encounter this oddity only with the 2.5 ng/ml concentration. Unfortunately, at this time, we cannot offer an explanation for this observation.

Based on observations during the course of these studies, we suggest that investigators use glass test tubes rather than polypropylene or polyethylene test tubes. This recommendation is made based on our observation of non-specific adsorption to plastic. Curcumin concentrations have differed by as much as 28% when incubated in plastic versus glass test tubes (Pak et al., unpublished observation).

5. Conclusion

In order to achieve the lowest possible quantifiable concentration with consistent reproducibility, three different liquid–liquid extraction methods were developed. These methods, along with the HPLC protocol, provide the sensitivity reported using a gradient method with the convenience of an isocratic system, which allows the processing of numerous samples within a short period of time. Authentic in vivo samples containing curcumin can be processed efficiently with higher sensitivity using this analytical method, resulting in more meaningful and appropriate pharmacokinetic analysis of curcumin concentration-time data. To date, this method provides the greatest sensitivity for quantitating curcumin from plasma samples.

Acknowledgements

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