March 2010 ISSN : 0974-7419 Volume 9 Issue 1

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 9(1) 2010 [93-101]

Development and validation of a new LC-MS/MS bio-analytical method for the determination of *Curcumin* in human plasma samples

Saurabh Arora[1], Sunny Chopra[1]*, Kanchan Kohli[1], Praveen Baliyan[2], Roop K.Khar[1]

[1]Faculty of Pharmacy, Jamia Hamdard, Hamdard University, New Delhi-110062, (INDIA)

[2]Arbro Pharmaceuticals Ltd., Kirti Nagar, New Delhi, (INDIA)

E-mail : sunnychopra82@gmail.com

Received: 9th December, 2009 ; Accepted: 19th December, 2009

ABSTRACT

The objective of current investigation is to develop a method that can be suitably applied for analysis of *curcumin* in human plasma. There is no sensitive bioanalytical method reported in literature for *curcumin* in human plasma yet. Hence it is of prime importance to develop a method for estimating *curcumin* in biological matrix with highest level of sensitivity for better estimate of pharmacokinetic parameters like $AUC_{0\rightarrow\alpha}$ (Area under curve from zero to infinity) and K_{el} (Elimination rate constant) in patients. A fast, sensitive and specific LC–MS/MS bio-analytical method has been developed and validated. We achieved not only high sensitivity of 10.08μg /ml as the Lower Limit of Quantification (LLOQ) but also showed dynamic linearity ranging from 10-500μg/ml (r^2=0.9991) with acceptable precision, accuracy and recovery (as per limits set by USFDA). Our novel method involves minimum extraction steps achieving an excellent recovery (more than 80% with Coefficient of Variation (CV) less than 10%). Sufficient stability was shown to allow for the completion of sample analysis in clinical trials. This validated rugged method led to analysis of large number of plasma samples in a very short span of time (due to lower retention time of 4-5 min).

KEYWORDS

Curcumin-polyphenol;
LC-MS/MS-liquid chromatography;
Mass spectroscopy.

INTRODUCTION

Curcumin

Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa* has a wide spectrum of biological and pharmacological activities[1,2]. Chemically, curcumin is bis-α,β-unsaturated β-diketone (Figure 1), which exhibits keto-enol tautomerism having predominant keto form in acidic and neutral sols and stable en-olic form in alkaline medium. Commercial *curcumin* contains approximately 77%

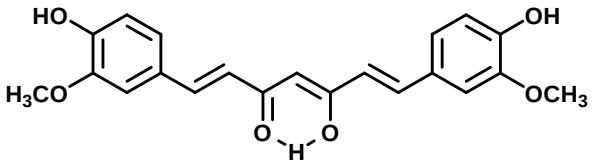

Figure 1 : Structure of *Curcumin*

diferuloylmethane, 17% demethoxycurcumin and 6% bisdemethoxycurcumin[3,5]. Traditionally *curcumin* has been used for many ailments[6,7], *curcumin* has been identified as the active principle of turmeric[8]. *Curcumin* has been shown to exhibit anti-inflammatory, antioxidant anti-inflammatory, antimicrobial and ant carcinogenic activities[9]. Various animal models or human studies proved that *Curcumin* is extremely safe even at higher doses[10-12].

Numerous analytical methods have appeared in the literature to analyze *Curcumin* on High Performance Liquid Chromatography (HPLC) but to analyze *Curcumin* in human plasma on LC MS/MS has not yet been reported. Methods using non-mass spectrometric detection are tedious and may not have the desired selectivity. In this paper, we present a highly sensitive LC-MS-MS method for the assay of *Curcumin* in human plasma. Particular effort was made to achieve higher method sensitivity and a high selectivity.

EXPERIMENTAL

Chemicals and reagents

Curcumin working standard was sourced from Dabur India Ltd. and Betamethasone used as Internal Standard (IS) was purchased from by Sigma-Aldrich (MO, USA). Drug free human plasma containing citrate phosphate dextrose adenine (CPDA) was collected from Prathma Blood center, Ahemdabad, India and Ethylene di-Amine tetra acetate (EDTA) plasma was sourced from Auriga Clinical Research, New Delhi. Formic acid, MeOH were purchased from Qualigens fine chemicals (a division of GSK Ltd, Mumbai, India) and used as received. MeCN was sourced from J.T. Baker.

Preparation of stock solutions

Primary stock sols for standards and Quality Control (QC) samples were prepared from separate weighing. The primary stock sols of *Curcumin* were prepared by weighing accurately about 25.63mg of *Curcumin* working standard which was transferred to a 25ml volumetric flask. This was dissolved in 5ml MeOH/Water mixture (50/50 v/v) and the volume (vol) was made up with the same to obtain 1mg /ml solution (sol). The above final concentration (conc) was corrected for *Curcumin* taking into account its potency and the actual amount weighed.

TABLE 1 : Depicting HQC, MQC, LQC and LLOQ

Spiking sol. conc.(µg/ml)	Vol.ofs piking sol.taken (ml)	Final vol.(ml)	Final conc. (µg/ml)	Spiked QC codes
50.1643	0.500	50	501.6426	HQC
30.0986	0.500	50	300.9856	MQC
10.0830	0.150	50	30.2490	LQC
1.0083	0.250	25	10.0830	LLOQ

Preparation of internal standard stock solution

About 18.7mg of Betamethasone working standard was weighed accurately. This was transferred to a 25ml volumetric flask which was dissolved in approximately 5ml MeOH/Water mixture (50/50, v/v) and the vol made up with the same to obtain 1mg /ml sol. The above final conc was corrected accounting for its potency and the actual amount weighed. All of the stock and working sols were stored below 4°C and protected from light.

Quality control (QC) samples

Spiking of plasma for quality control samples

Accurate vol of each of the above described stock dilutions of *Curcumin* were transferred to a volumetric flask and the vol was made up with plasma to achieve the following quality control samples which were labeled as High Quality Control Concentration (HQC), Medium Quality Control Concentration (MQC), Low Quality Control Concentration (LQC), and LLOQ respectively (TABLE 1). Aliquot (1.5ml) of each quality control spiked samples were taken into polypropylene-capped tubes which were frozen below -50°C until analysis.

Instrumentation

A liquid chromatographic system API-3000 LC-

TABLE 2 : Mobile phase gradient

Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0	550	90	10
1	0.5	550	90	10
2	0.8	550	10	90
3	4.0	550	10	90
4	4.2	550	90	10
5	7	550	90	10

MS/MS System consisting of PerkinElmer LC pump; PerkinElmer Auto Sampler and C_{18} column (Merck, particle size 3µm and Internal Diameter-4.67mm) was used for the separation. The LC-MS-MS system consisting of atmospheric pressure heated nebulizer source (API-3000) with triple-quadrupole tandem mass spectrometer (Applied Biosystems) was used for quantitative determination of *Curcumin* in plasma. Data integration was performed with Analyst™ 1.4.1 software version supplied by Applied Biosystem.

Chromatographic conditions

The mobile phase consisting of a mixture of Solvent A (0.1% (v/v) formic acid sol in water) and Solvent B (0.1% (v/v) formic acid sol in MeOH) was delivered at a flow rate of 0.55ml/min with a gradient shown in TABLE 2. The column was thermostatically controlled to a temperature of 40°C and sample cooler temperature was maintained at 10°C. The injection vol was 20µl.

Mass spectrometric conditions

A triple-quadrupole mass spectrometer (MS-MS) was used with ESI source and channel electron multiplier (CEM) detector in positive ion detection mode. In ESI source, the temperature of the heater in the source was maintained at 400°C with nebulizer gas at 8 psi (zero air) for the better vaporization of HPLC eluent. For the transition of ions from atmosphere to vacuum region, curtain gas (ultra high purity nitrogen) was served at 6 psi for the effect of collisional induced dissociation (CID) in the curtain plate region. For the precursor ion fragmentation, collisional gas (ultra high purity nitrogen) was served at 5 psi for the effect of collisional activated dissociation (CAD) in the collision cell. MRM mode was used for scanning throughout this study. The transitions selected were *m/z* 369.10 → *m/z* 285.20 and *m/z* 393.40 → *m/z* 373.00 for *Curcumin* and IS respectively, with a dwell time of 200 ms per transition.

Human plasma extraction

One set of calibration curve standards, three sets each of quality control samples and plasma samples were withdrawn from the deep freezer and allowed to thaw at room temperature. Samples were than subjected to vortex for 5 min. From this 400µl of sample was taken in eppendorff's tubes and subsequently 20µl of 4.0168µg/ml IS was added to it. This was again subjected to vortex for 15 s. To this 800µl of MeCN was added which was again vortex mixed for 5 min and centrifuged at 4000rpm for 10 min. The clear portion from the sample was transferred into another centrifuge tube and 1 ml of ethyl acetate was added to it. This was than vortex mixed for 5 min and centrifuged at 4000 rpm for 10 min. The upper layer was transferred into a dry test tube & evaporated under nitrogen at 40°C. The sample was reconstituted with 50% MeOH and injected on LC-MS/MS System.

Method validation

The method was validated in terms of selectivity, linearity, sensitivity, precision, accuracy, recovery and stability according to the guidelines issued by USFDA for the validation of bioanalytical methods[13].

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Blank plasma samples from six different volunteers were tested for the presence of endogenous compounds (that might interfere with anal) using the proposed extraction procedure at the same chromatographic/mass-spectroscopic conditions. Each blank sample should be tested for interference, and selectivity was ensured at the LLOQ.

The results were then compared to meet regulatory acceptance criteria.

Calibration curve, linearity and sensitivity

A calibration curve was constructed using a double-blank sample (a plasma sample without *Curcumin* and IS) and eight calibration samples covering the whole range of conc (10-500µg/ml). Conc of *Curcumin* were calculated from these area ratios using the calibration curve. The linearity of the calibration curve was also calculated, and a correlation coefficient (r^2) of 0.999 or better was deemed satisfactory. LLOQ was defined as the lowest conc with a CV and relative standard deviation (R.S.D.) of ≤20%.

Accuracy and precision

The accuracy of an analytical method describes the closeness of mean test results obtained by the method

to the true conc of the anal. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy was measured using five determinations per conc (taking four conc). The deviation of the mean from the true value serves as the measure of accuracy. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.

Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous vol of biological matrix. Intra- and inter-day assay precisions were determined as coefficient of variance (CV), and intra- and inter-day assay accuracies were expressed as percentages of the theoretical conc, as accuracy (%) = (calculated conc / nominal conc)×100. Intra-day assays were performed using five replicates during 1 day and inter-day assays were performed on six separate days. The acceptance criterion recommended by USFDA for each back-calculated standard conc was a 15% deviation from the normal value except at the LLOQ, which was set at 20%.

Matrix effect

To assess the matrix effect, standard conc sol of *Curcumin* (taken at LLOQ) was processed. Two groups of six blank plasma samples of different sources were spiked with the above standard sol and the clear supernatants of 500µl were injected. For each lot, duplicate samples were analyzed. The CV of the peak area for *Curcumin*/peak area of IS was calculated and inter-lot matrix variability was determined.

Extraction recovery

For evaluation of recovery three sets of QC samples (30.25, 300.99, 501.64µg/ml, $n = 6$) were prepared and extracted by the preparative procedure described above with IS. The recovery of extracted samples was compared with dilution in the same way in mobile phase as the actual dilution during extraction. The non-extracted samples were injected into the system. Peak areas of the non-extracted samples were compared with extracted samples from plasma and acceptance criteria being CV to be less than 20% amongst the three conc to establish drug recovery.

Stability

The stability of *Curcumin* was assessed by analyzing QC samples (at low and high level conc with six determinants for each) exposed to different temperatures for different times. QC samples were prepared in sufficient vol to allow multiple replicates ($n = 6$) at each test condition. Results were compared with those of freshly prepared QC samples, and percentage conc deviations were calculated. Stability was calculated as the difference from the freshly prepared samples. The protocol for the stability study included short- and long-term stability.

Freeze and thaw stability

Analyte stability was determined after three freeze and thaw cycles. Six aliquots at each of the low and high conc were stored at the intended storage temperature for 24 hs and thawed at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hs under the same conditions. The freeze–thaw cycle was repeated two more times, and then analyzed on the third cycle.

Short term temperature stability

Six aliquots each of the low and high conc were thawed at room temperature and kept at this temperature for 6 h and analyzed.

Dilution integrity

The effect of diluting plasma was assessed by preparing QC samples ($n = 6$) with *Curcumin* conc at 2 times and 4 times dilution.

Long term stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability was determined by storing at least six aliquots of each of the low and high conc under the same conditions as the study samples for a period of 20 days.

Post preparative stability

The stability of processed samples, including the resident time in the injector was determined. The stability of the drug was assessed for a period of 24 hrs.

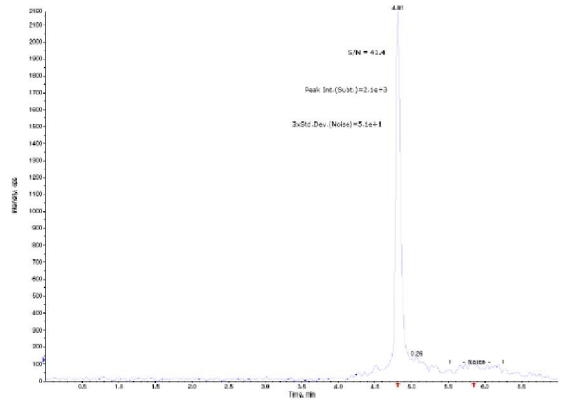

Figure 2 : Chromatogram of *Curcumin* at LLOQ depicting s/n ratio

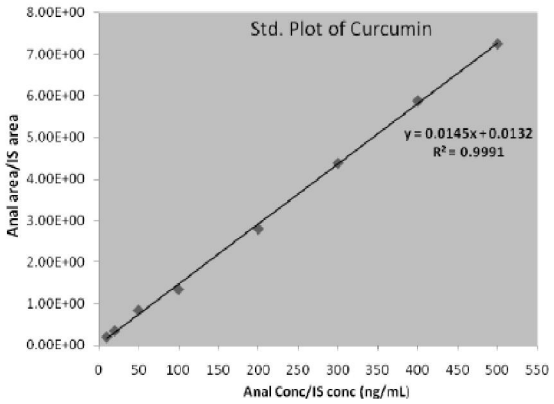

Figure 3 : Linearity curve of *Curcumin*

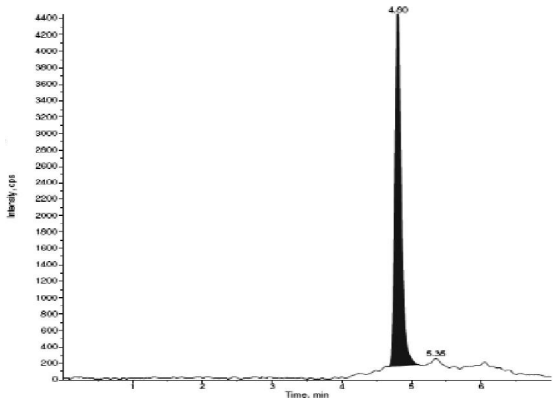

Figure 4 : Representative chromatogram of *Curcumin* at conc of 20µg/ml

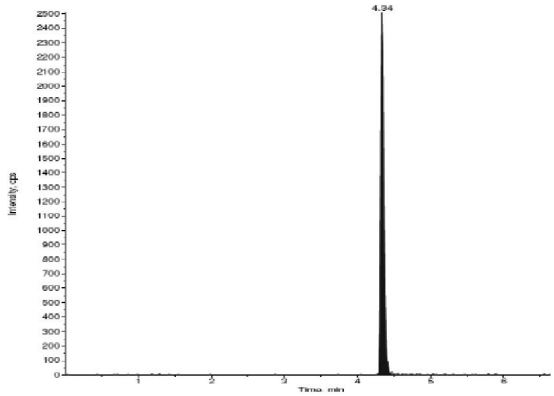

Figure 5 : Chromatogram of IS at a conc. of 80.33µg/ml

TABLE 3 : Linearity data of *Curcumin* in conc. range of 10-500µg/ml

Curcumin conc. (µg/ml)	Analyte Peak area	IS peak area	Cal.Conc. (µg/ml)	Accuracy	Analyte Area/IS Area
10	6.90E+03	3.64E+04	10.0	100.0	1.90E-01
20	1.06E+04	3.14E+04	20.4	102.0	3.38E-01
50	2.83E+04	3.40E+04	55.1	110.0	8.32E-01
100	4.53E+04	3.39E+04	90.2	90.2	1.34E+00
200	8.59E+04	3.08E+04	188.0	94.0	2.79E+00
300	1.48E+05	3.38E+04	305.0	102.0	4.38E+00
400	1.81E+05	3.08E+04	387.0	96.6	5.88E+00
500	2.50E+05	3.45E+04	525.0	105.0	7.25E+00

RESULTS

Validation and assay performance

Linearity

The standard plot of *Curcumin* was plotted and linearity was observed in the range of 10-500µg/ml (Internal standard at conc of 1µg/ml) with an r^2 value of 0.9991 (TABLE 3), which was found to be well within the acceptable limit as shown in figure 3. Signal-to-noise ratio greater than 10 was achieved at the LLOQ (10.08µg/ml) as shown in figure 2. A representative chromatogram of *Curcumin* at a conc of 20µg/ml is shown in figure 4.

Accuracy

Three consecutive precision and accuracy batches were completed. Each batch included calibration standards freshly prepared and extracted from human plasma, a blank and a zero (replicate $n = 5$ for accuracy and n = 6 for precision). QC samples were prepared at LLOQ (10.08µg/ml), low (30.25µg /ml),

Full Paper

TABLE 4 : Accuracy at n=5

Conc. µg/ml	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Mean	S.D.	%C.V.	Accuracy
10.08	11.20	8.36	8.66	8.66	11.10	9.60	1.424	14.843	95.20
30.25	26.10	28.80	31.30	28.10	28.40	28.54	1.861	6.520	94.35
300.99	293.11	289.08	319.60	285.60	290.01	295.48	13.747	4.652	98.17
501.64	503.21	509.52	509.04	490.18	481.29	498.65	12.450	2.497	99.40

TABLE 5 : Intraday precision at 5 different times on same day

Type	Conc (µg/ml)	Cal.Conc. 1	Cal.Conc. 2	Cal.Conc. 3	Cal.Conc. 4	Cal.Conc. 5	Cal.Conc. 6	Avg	Acc	S.D	RSD
LLOQ	10.08	10.16	9.36	8.96	8.56	10.08	11.05	9.70	96.18	0.910	9.381
LQC	30.25	28.15	28.06	28.39	31.32	28.07	28.84	28.80	95.21	1.267	4.398
MQC	300.99	284.58	280.81	292.83	290.63	318.79	276.60	290.71	96.58	15.019	5.167
HQC	501.64	490.45	484.51	513.26	517.67	462.99	475.87	490.79	97.84	21.273	4.334

TABLE 6 : Interday Precision on 6 different days

Type	Conc. (µg/ml)	Conc. day 1	Conc. day 2	Conc. day 3	Conc. day 4	Conc. day 5	Conc. day 6	Average	Accuracy	S.D.	RSD
LLOQ	10.08	11.32	7.82	11.06	11.26	13.03	11.05	10.92	108.36	1.695	15.522
LQC	30.25	30.25	29.35	25.84	28.58	33.25	30.74	29.67	98.07	2.459	8.287
MQC	300.99	292.09	316.12	320.83	301.55	299.38	328.56	309.76	102.91	14.168	4.574
HQC	501.64	512.81	525.50	522.28	495.63	515.49	556.68	521.40	103.94	20.175	3.869

TABLE 7 : Recovery of IS

	Low	Medium	High
Conc. µg/ml	21.45	60.62	80.33
	97.82	97.62	102.82
	103.58	96.24	97.15
Recovery	96.46	96.83	94.05
	95.54	101.97	100.11
	97.19	97.85	98.07
	97.05	102.66	101.82
Mean	97.94	98.86	99.00
S.D.	2.868	2.744	3.242
% C.V.	2.928	2.776	3.275

TABLE 8 : Recovery of analyte

	Low	Medium	High
Conc. µg/ml	30.25	300.99	501.64
	96.82	95.26	94.62
	102.68	96.31	97.11
Recovery	94.33	95.69	94.05
	95.50	102.32	101.19
	97.21	97.91	98.07
	96.35	102.54	103.82
Mean	97.15	98.34	98.14
S.D.	2.899	3.295	3.788
% C.V.	2.984	3.351	3.860

medium (300.99µg/ml) and high (501.64µg /ml) conc levels.

In this study it was found that % CV at LLOQ was 14.8428 whereas % C.V. at 30.25,300.99 and 501.64µg/ml was found to be 6.5204, 4.6523 and 2.4968 respectively (TABLE 4) which was within limit set by USFDA.

Precision

Intraday precision

Intraday precision prevailed as the RSD values obtained were with in limits there by depicting that Intrabatch reproducibility was there (as shown in TABLE 5).

Interday precision

Interday precision (done for 6 days) was found within limits with RSD value at LLOQ being 15.5221 and 8.287, 4.574 and 3.869 at conc of 30.25, 300.99 and 501.64 respectively as shown in TABLE 6.

Matrix effect

In this study the % CV was found to be 4.6822 (should be ≤15%) which proved the study to be free of matrix effect.

TABLE 9 : Freeze and thaw stability

	Initial		Cycle 1		Cycle 2		Cycle 3	
	(LQC) 30.25	(HQC) 501.64	(LQC) 30.25	(HQC) 501.64	(LQC) 30.25	(HQC) 501.64	(LQC) 30.25	(HQC) 501.64
Conc. (µg/ml)	30.96	476.57	29.96	463.57	31.71	500.74	27.33	483.01
	29.64	488.92	28.64	488.92	32.22	454.63	28.24	449.17
	29.77	500.95	30.77	500.95	29.93	450.27	26.54	483.19
	30.00	501.69	30.00	501.69	28.26	466.82	28.52	460.17
	30.06	487.12	30.06	487.12	29.71	466.17	30.20	479.51
Mean	30.09	491.05	29.89	488.45	30.36	467.73	28.17	471.01
S.D.	0.517	10.497	0.772	15.429	1.604	19.806	1.381	15.484
%CV	1.718	2.138	2.584	3.159	5.284	4.234	4.902	3.287
Acc.			99.34	99.47	100.92	95.25	93.62	95.92

TABLE 10: Short term stability

Time	0 hr		4 hrs		6 hr	
	LQC	HQC	LQC	HQC	LQC	HQC
Conc (µg/ml)	32.23	494.17	30.21	480.62	25.65	469.60
	26.95	520.10	31.46	512.32	29.13	509.77
	29.69	491.27	29.66	462.27	32.42	495.93
	29.33	436.41	31.38	510.14	28.04	447.77
	29.90	520.26	28.38	488.02	27.21	531.01
	30.41	469.28	27.57	500.68	28.09	484.76
Mean	29.75	488.58	29.78	492.34	28.42	489.81
S.D.	1.712	32.032	1.576	19.214	2.274	29.444
% C.V.	5.755	6.556	5.294	3.903	8.002	6.011
Accuracy			100.09	100.77	95.54	100.25

TABLE 11 : Dilution integrity

Dilution	2 times diluted(501.64)	4 times diluted (250.82)
Conc. (µg/ml)	481.88	238.03
	487.79	225.88
	504.52	268.57
	494.11	260.70
	510.04	254.12
	478.58	222.56
Mean	492.82	244.98
S.D.	12.513	18.987
% C.V.	2.539	7.751
Accuracy	98.242	97.670

Recovery of internal standard and analyte

The recovery of IS (97.940, 98.862 and 99.003% at 21.45, 60.62 and 80.33µg/ml respectively) and analyte (97.148, 98.338 and 98.143% at 30.25, 300.99 and 501.64 respectively) achieved was not only very high but also consistent, precise, and reproducible (TABLE 7 and 8). Figure 5 showing a Chromatogram of IS at conc of 80.33µg/ml.

Stability

Freeze and thaw stability

The samples studied were found to be stable after 3 cycles of freeze and thaw stability (three conc taken) and CV and accuracy compared to freshly prepared samples were found to be in acceptable limits (as shown in TABLE 9).

Short term stability

This study showed that samples were stable at room temperature till 6 hrs and so they can be kept at room temperature for above mentioned period without loosing sample integrity (TABLE 10).

Dilution integrity

The back-calculated conc for dilution controls were in agreement with the theoretical (prepared) conc. As shown in TABLE 11 the accuracy was 98.242% and 97.670% with a % CV of 2.539%.and 7.751% after 2 and 4-fold dilution with plasma respectively. These results suggest that samples with conc greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Long term stability

Long term stability up to 20 days was found satisfactory with accuracy level of 99.2812 and 99.4548 at 30.25 and 501.64µg/ml when compared to freshly prepared samples as shown in TABLE 12.

Post preparative stability

Post preparative stability (after sample preparation)

Full Paper

TABLE 12: Long term stability

Time	0 DAY		20 DAYS	
	LQC	HQC	LQC	HQC
Conc. (µg/ml)	32.23	494.17	30.11	462.38
	30.95	520.10	31.16	515.99
	29.69	491.27	29.51	490.92
	29.33	496.41	30.46	500.10
	29.90	520.26	28.99	511.50
	30.41	469.28	30.96	494.28
Mean	30.42	498.58	30.20	495.86
S.D.	1.054	19.335	0.838	19.056
%C.V.	3.466	3.878	2.776	3.843
Accuracy			99.28	99.45

TABLE 13 : Post preparative sample stability

Time	0 HR		6 HR		12 HR		24 HR	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Conc. (µg/ml)	32.82	486.13	30.47	472.31	27.56	426.95	29.30	451.34
	32.09	458.62	29.77	487.69	31.36	479.28	30.96	463.16
	31.43	496.19	30.13	487.28	28.22	489.18	25.86	484.11
	33.20	495.71	30.15	522.74	29.47	482.69	29.82	491.80
	28.64	495.38	31.11	471.04	30.91	495.38	28.70	487.33
	29.53	503.65	27.35	423.99	29.32	494.98	32.14	484.40
Mean	31.28	489.28	29.83	477.51	29.47	478.08	29.46	477.03
S.D.	1.832	16.016	1.295	32.179	1.475	25.865	2.150	16.006
%C.V.	5.856	3.273	4.342	6.739	5.004	5.410	7.296	3.355
Accuracy			95.36	97.59	94.20	97.71	94.18	97.50

was found to be with in limits till 24 hrs when compared with freshly prepared samples (TABLE 13).

DISCUSSION

Quantification of drugs in biological matrices by LC/MS/MS is becoming more demanding due to the improved sensitivity and selectivity of this technique[14]. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. The analysis of *Curcumin* from human plasma is of major interest in pharmaceutical research as sensitive and rugged method is yet to be achieved.

Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that addition of formic acid increased signal without affecting noise level and best combination of MeOH and aqueous sol of formic acid was rationally optimized. The high proportion of solvent eluted the analyte and the IS at retention times of 4.74 and 4.34 min, respectively and at the same time no interference from endogenous compounds were found. A flow rate of 550µl /min produced good peak shapes and permitted a run time of 6 min .Column performance was excellent with no back pressure or LC related problem found even during subject sample analysis on day to day basis, major reason being column temperature optimized at 45°C. Recoveries of the analyte and IS were excellent as protein precipitation involved minimum number of steps, and method was consistent, precise and reproducible. Therefore, the assay had proved to be robust in high-throughput bioanalysis.

Choosing the appropriate IS is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Therefore, after trials with different IS, we finally opted for Betamethasone, reason being that it is a stable molecule with recoveries and extraction similar to that of *Curcumin*. In addition, its retention behavior was found similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. The importance of including the evaluation of matrix effect in any LC/MS/MS method was outlined in papers by Benijts et al.[15]. Their data strongly emphasize the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this assay were performed with matrices obtained from different individuals (n=6). As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

Curcumin was stable (±10% change in measured conc compared to conc in stability samples at 0 hr) in human plasma following three freeze/thaw cycles (freeze-thaw stability); For bench top stability (short

term) samples after being frozen, thawed and kept at room temperature (20°C) for at least 6 hr and for at least 20 days when studied for long term stability. *Curcumin* was stable in extracts for at least 24 hr when stored in the auto injector to support post processing in-injector stability. Additionally, *Curcumin* was also stable for up to at least 20 days in MeOH stored in the fridge (4°C) and supports stock sol stability.

CONCLUSION

For exploratory as well as developmental research it is always advocated to have a simple cost effective rugged method which will ensure day to day rapid analysis of drugs from biological matrices. A method has been described for quantification of *Curcumin* in human plasma by LC/MS/MS. The precision and accuracy of the method met the criteria laid down in Guidance for Industry, Bioanalytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Sufficient stability was shown to allow for the completion of sample analysis in clinical trials. This newly developed, validated method was more sensitive, selective precise, rugged with dynamic linear range and therefore finds successful application to support a pharmacokinetic study.

ACKNOWLEDGEMENTS

The above research work has been carried out by the funding provided by Department of Science and Technology, Government of India and Arbro Pharmaceuticals Ltd., New Delhi, India.

REFERENCES

[1] S.Balasubramanian, R.L.Eckert; J.Biol.Chem., **279**, 24007 **(2004)**.
[2] E.M.Bruzell, E.Morisbak, H.H.Tonnesen; Photochem. Photobiol.Sci., **4**, 523 **(2005)**.
[3] B.B.Aggarwal, C.Sundaram, N.Malani, H.Ichikawa; Adv.Exp.Med.Biol., **595**, 1 **(2007)**.
[4] R.A.Sharma, A.J.Gescher, W.P.Steward; Eur.J. Cancer, **41**, 1955 **(2005)**.
[5] S.K.Vareed, M.Kakarala, M.T.Ruffin, J.A.Crowell, D.P.Normolle, Z.Djuric, D.E.Brenner; Cancer Epidemiol Biomarkers Prev., **17**, 1411 **(2008)**.
[6] H.Y.Yuan, S.Y.Kuang, X.Zheng, H.Y.Ling, Y.B.Yang, P.K.Yan, K.Li, D.F.Liao; Acta Pharmacol. Sin., **29**, 555 **(2008)**.
[7] S.Yang, D.Zhang, Z.Yang, X.Hu, S.Qian, J.Liu, B.Wilson, M.Block, J.S.Hong; Neurochem. Res., **(2008)**.
[8] M.A.Azuine, J.J.Kayal, S.V.Bhide; J.Cancer Res. Clin.Oncol., **118**, 447 **(1992)**.
[9] C.C.Araujo, L.L.Leon; Mem.Inst.Oswaldo Cruz, **96**, 723 **(2001)**.
[10] A.L.Berger, C.O.Randak, L.S.Ostedgaard, P.H.Karp, D.W.Vermeer, M.J.Welsh; J.Biol.Chem., **280**, 5221 **(2005)**.
[11] H.Chen, Z.S.Zhang, Y.L.Zhang, D.Y.Zhou; Anticancer Res., **19**, 3675 **(1999)**.
[12] Y.G.Zhu, X.C.Chen, Z.Z.Chen, Y.Q.Zeng, G.B.Shi, Y.H.Su, X.Peng; Acta Pharmacol.Sin., **25**, 1606 **(2004)**.
[13] F.D.A., (CDER), 'Guidance for Industry Bioanalytical Method Validation', Available at http:/ /www.fda.gov/cder/guidance/index.htm, 1st Dec., **(2009)**.
[14] T.A.Mary Pelzer, Wenbao Li, Xiangyu Jiang, Naidong Weng; J.Liquid Chrom.& Rel.Technol., **25**, 1019 **(2002)**.
[15] R.D.Tom Benijts, Willy Lambert, Andre De Leenheer; Journal of Chromatography A, **1029**, 153 **(2004)**.