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QUANTIFICATION OF CURCUMIN IN HUMAN PLASMA BY UPLC-MS/MS METHOD

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ABSTRACT

An accurate, specific ultra performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) method for the quantitative determination of curcumin using the human plasma. Curcumin D6 is used as an internal standard (IS). The compound was extracted from the plasma using Liquid-Liquid extraction technique. The organic solvent was evaporated and reconstituted in the mobile phase. The experiment was done using ACQUITY UPLC BEH 1.8 µm (2.1mm*100mm) column and time of flight mass spectrometer was used for the detection of compound equipped with electro spray ionization in Negative mode. The Mass lynx 1.4 software is used to obtain the data of the analysis. The mobile phase used here in combination of Acetonitrile and Buffer in the ratio of (90:10, v/v) with 0.1% formic acid and isocratic elution was used.The total run time was 2.50minthe analysis done for quantification using the MRM (Multiple Reaction Monitoring) with mass / charge (m/z) ratio parent ion of curcumin was 367.16 and curcumin D6 was 373.20, daughter ion of curcumin 148.99 and curcumin D6 was 176.180, the dwell time of curcumin 0.100 and curcumin D6 was 0.100, cone voltage of curcumin was 30 and curcumin D6 was 30. The retention time of curcumin and IS 1.45 and 1.4 mins respectively. The Bio analytical methods are accurate and

reliable to determine these drugs at lower concentrations. Selectively Liquid Chromatography-Mass Spectrometry method to quantitate Curcumin in K_2EDTA.Human plasma over the concentration range 0.4180 to 72.2360 ng/mL was successfully validated. This method is suitable for sample analysis to support bio-equivalence/bioavailability studies involving formulations of Curcumin. By ultra-performance Liquid chromatography UPLC–MS/MS is currently considered as the best choice. Curcumin is quantifiedthrough UPLC-MS/MS and it has high specificity, more sensitivity, and increased rapidity.

Keywords: Curcumin, UPLC–MS/MS, K_2EDTA, Quantification, Bio-equivalence, Bioavailability

INTRODUCTION

Bio analysis is a part in analytical chemistry focusing the quantitative measurement of drugs. The metabolites of biological systems like blood, plasma, serum, urine or tissue extracts etc., are widely used in bio-analysis. Bio analytical methods are used to determine the drug at very lower concentration that will be accurate and reliable. This provides improvements in technology and analytical development.

Generally High-Performance Liquid Chromatography is used for identification, separation and quantification of mixture compounds **[1]**. The principles of UPLC and HPLC are same but UPLC has many advantages and more developments compared to HPLC the important advantage is mainly considered are speed, quality and cost of analysis **[2]**. High sensitivity in analysis, decreasing the consumption of solvents during analysis andit has high speed in analysis **[3]**. The most important cause for the usage of UPLC than HPLC is Sensitivity it is increased up to 3 to 5 times **[4]**. **UPLC** is coupled with mass spectrometer for the better results m/z ratio is determined through TOF. **Time-of-flight mass spectrometry** (TOFMS) is a method of mass spectrometry in which an mass/charge ratio is determined by a time of flight measurement **[5, 6]**.

Curcumin is a yellow colour compound it can be obtained from the plantsof Curcuma *longa* species. It is otherwise known as Indian saffron belongs to the family of zingiberoside. Curcumin is chemically 1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. Molecular Formula of curcumin is $C_{21}H_{20}O_6$, molecular weight is368.39 and its Melting pointis 183 °C (361°F; 456 K).Curcumin is a beta-diketone the hydrogens are substituted to feruloyl groups. The Dyestuff present in the root of Curcuma longa. It also contains

polyphenol, a beta-diketone, an enone, a diarylheptanoid and an aromatic ether. The structure of curcumin shown in **Figure 1**.

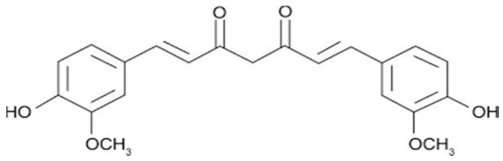

Figure 1: Chemical structure of curcumin

Curcumin has many biological actions such as Antitumor, Anti-inflammatory, Antivirus, neuroprotective, Anti-acidogenic, Radioprotective Wound healing-metastatic, Anti-bacterial, fungal, Arthritis, Antioxidation, and Anti-human immunodeficiency virus (HIV) **[7, 8]**. The number of animal models or human studies concluded that curcumin is very safe even when the doses are very high than normal. The solubility profile, chemical stability, absorption, and metabolism of the compound have been provided evidence was that compound has poor oral bioavailability. It undergoes rapid metabolic conjugation generating mainly curcumin glucuronide and curcumin sulfate. Therefore, availability of free curcumin in plasma is very less.The quantification of curcumin plays a vitalrole in Analytical methods.

EXPERIMENT

Chemical and reagents:

Acetonitrile [HPLC grade], Ammoniumformate [AR grade], Ethyl Acetate [AR grade], n-Hexane [AR grade], Formic acid [AR grade], Water [HPLCgrade], Curcumin [working standard], Curcumin D6[internal standard], K_2EDTA, RIAVials, PDtips, Volumetricflasks, Reagentbottles.

UPLC-MS/MS Conditions:

The experiment was done using ACQUITY UPLC BEH 1.8 µm(2.1mm*100mm) column and time of flight mass spectrometer was used for the detection of compound equipped with electro spray ionization in Negative mode. The Mass lynx 1.4 software is used to obtain the data of the analysis. The mobile phase used here in combination of Acetonitrile and Buffer in the ratio of (90:10, v/v) with 0.1%formic acid and isocratic elution was used.Injection volume was 10µl and flow rate at the speed of 0.200ml/minute. The column oven used during analysis was Acquity Column Oven, supplied by Waters.inc at temperature of 35°±10°C.Auto sampler was used automization of sample and the auto sampler temperature was 10°±5°C.The pump used Binary Solvent Manager (BSM), Waters. The total run time was 2. 50min.The retention time of curcumin and IS 1.45 and 1.4 mins respectively.

The mass spectrometer was optimized using the tuning parameters as source condition are as follows the source temperature is 150-degree Celsius,

Capillary voltage was 3.00kv, the Cone voltage was30.00v, the cone gas flow was 50, the Desolvation temperature was 500 and desolvation gas flow was 800

The detector was performed in (ESI) (-) negative mode of ionization and the analysis done for quantification using the MRM (Multiple Reaction Monitoring) with mass / charge (m/z) ratio parent ion of curcumin was 367.16 and curcumin D6 was 373.20, daughter ion of curcumin 148.99 and curcumin D6 was 176.180, the dwell time of curcumin 0.100 and curcumin D6 was 0.100, cone voltage of curcumin was 30 ad curcumin D6 was 30.

Preparation of Buffer 5mM Ammonium Formate

Weigh about 315.30 mg of Ammonium Formate and transfer into 1000 ml reagent bottle containing about 500 mL of water and dissolve. Further makeup the volume with water Mix well. Filter through 0.2µm nylon membrane filter and sonicate in ultrasonic bath for few minutes.

Preparation of diluent: Acetonitrile: water (50:50%, v/v)

Transfer 250 mL of Acetonitrile into 500 ml reagent bottle and add 250 ml of Water. Mix well and filter through 0.2 µm nylon membrane filter and sonicate in ultrasonic bath for few minutes. complete the Solution Preparation.

Preparation of mobile phase: Acetonitrile: Buffer: (90:10, v/v) with 0.1% Formic Acid

Transfer about 900 ml of Acetonitrile and 100 mL of Buffer into 1000 ml reagent bottle and add 1mL formic acid. Mix well and filter through 0.2 µm nylon membrane filter and sonicate in ultra sonicate bath for few minutes.

Preparation of strong wash solution: Acetonitrile: Water (90:10, v/v) with 0.1% Formic Acid

Transfer about 900 ml of Acetonitrile and 100 ml of Water into 1000 ml reagent bottle and add 1ml formic acid. Mix well and filter through 0.2 µm nylon membrane filter Sonicate in ultrasonic bath for few minutes.

Preparation of weak wash solution: Acetonitrile: Water (50:50 v/v) with 0.1%Formic Acid

Transfer about 500 ml of Acetonitrile and 500 ml of Water into 1000 ml reagent bottle and add 1 mL formic acid. Mix well and filter through 0.2 µm nylon membrane filter. Sonicate in ultrasonic bath for few minutes.

Preparation of extraction solvent: [ethyl acetate: n- hexane (90:10, v/v)

Transfer about 900 mL of Ethyl Acetate.100 mL of n Hexane into 1000 ml reagent bottle and mix well.

Preparation of beta-glucuronidase: (5 mg/ml)

Weigh about 50 mg of beta-Glucuronidase and transfer into 10mL volumetric flask. Add 5 ml of water to dissolve and make up to the volume with water.

Preparation of sulfatase: [1mg/ml]

Weigh about 10 mg of sulfate and transfer into 10mL volumetric flask. Add 5 ml of water to dissolve and make up to the volume with water.

INTERNAL STANDARD DILUTION

Prepare stock dilution for internal standard CURCUMIN D6 in the concentration range of 100 ng/ml using diluent as the internal standard **(Shown in Table 1)**.

Table 1: Internal Standard Dilution

MOLE. NAME	STOCK COCENTRATION [µg/ml]	STOCK ALIQUOT [mL]	DILUENT ADDED [mL]	FINAL VOLUME [mL]	FINAL COCENTRATION [µg/ml]
Curc D6	1000.000	0.040	19.960	20.000	2.0000

Preparation of Analytical solution Preparation of Curcumin stock solution for CC (1 mg/ML)

Weigh accurately about 2 mg of Curcumin and transfer into 2 mL volumetric flask Add 0.200 ml of DMSO and 1 ml Acetone-M to dissolve and make up to the volume with Acetone-Calculate the final concentration of Curcumin in µg/ml as follows:

$$\frac{\boldsymbol{Weight\ of\ curcumin[mg]}}{\boldsymbol{2ml}} \boldsymbol{X} \frac{\boldsymbol{Potency\ (as\ is\ basis)}}{\boldsymbol{100}} \boldsymbol{X} \frac{\boldsymbol{M_1}}{\boldsymbol{M_2}} \boldsymbol{X\ 1000}$$

Where, M_1 is the molecular weight of Curcumin (free) and M_2 is the molecular weight of Curcumin.

Preparation of internal standard curcumin D6 (1mg/ml)

Weigh accurately about 2mg of curcumin D6. Transfer into a 2ml of volumetric flask Add 0.2 ml of DMSO and 1ml of Acetone-M to dissolve Make up to the volume with Acetone –M Calculate the final concentration of Curcumin D6 in µg/ml as follows:

$$\frac{\boldsymbol{Weight\ of\ curcuminD6[mg]}}{\boldsymbol{2ml}} \boldsymbol{X} \frac{\boldsymbol{Potency\ (as\ is\ basis)}}{\boldsymbol{100}} \boldsymbol{X} \frac{\boldsymbol{M_1}}{\boldsymbol{M_2}} \boldsymbol{X\ 1000}$$

Where, M_1 is the molecular weight of Curcumin (free) and M_2 is the molecular weight of Curcumin.

Curcumin stock dilution for calibration curve

Prior spiking, prepare mixed stock dilutions of curcumin by using diluent in the concentration range **(shown in Table 2)**.

PROCEDURE:

Withdraw the required number of quality samples and calibration curve

standards for method validation. From ultra-low temperature freezer/Low temperature Freezer and allow them to thaw a room temperature. Make Vortexingthe thawed samples for the confirmation of complete mixing of contents. Add 50 µl of internal standard solution (Curcumin D6, 2µg/mL) into all respectively labeled empty RIA vials except blank. Pipette 400 µl of plasma samples into the respectively labelled RIA vials. Add 100 µl of buffer into all the samples and vortex. Add 2.5 mL of Extraction Solvent into all the samples and cap them. Keep all the samples on vibramax at 2000 rpm for 10 minutes. Centrifuge the samples at 3500 rpm for 5 minutes at 2-8°C in a refrigerated centrifuge. Transfer 2.0 ml of supernatant into respectively labeled RIA Vials.Dry all the samples under nitrogen evaporator at 40°C and 15 psi pressure. Reconstitute the dried residue with 100 µl of Buffer and vortex. Add 50 µl of Beta-Glucuronidase and add 50µl of Sulfatase into all the samples and vortex. Keep all the samples at room temperature for 1hour.Add 400 µl of water into all the samples and vortex. Add 2.5 mL of Extraction Solvent into all the samples and cap them. Keep all the samples on vibramax at 2000 mm for 10 minutes. Centrifuge the samples at 3500 rpm for 5 minutes at 2-8degree Celsius in a refrigerated centrifuge. Transfer 2.0 ml of supernatant into respectively labeled RIA vials. Dry all the samples under nitrogen evaporator at 40degree Celsius and 15 psi pressure. Reconstitute the dried residue with 150 µl of Mobile Phase and vortex. Transfer all the samples into respectively labeled auto-injector vials. Load the processed samples into LC-MS/MS.

NOTE: Process all steps under monochromatic light condition or low yellow light.

VALIDATION PARAMETERS

Precision and Accuracy

Assay precision and accuracy values were by analysing six replicates each of LQC, INTQC, MQC, and HQC samples. Consecutive numbering of the QC samples may not be reciting in the tables as some of the samples have been randomly used to perform various validation parameters.

Accuracy

The accuracy of the assay is defined as absolute value of the ratio of calculated mean values of the quality control samples to their respective nominal values expressed as percentage.

Precision

The precision of the assay was measured by the percentage co-efficient of variation over the concentration range of LQC, INTQC, MQC and HQC sample of

Curcumin during the course of method validation **(Shown in Table 3)**.

Linearity

Linearity established by preparing an eight-point standard calibration curve in K_2EDTA Human plasma covering the Curcumin concentration ranged from 0.4180 to 72.2360 ng/mL using Curcumin D6 as internal standard. Precision and accuracy batch was analysed in this range. Calibration curves were calculated by least square linear regression analysis of the response ratios (Analyte/Internal standard) in the calibration standards using a weighting factor of $1/X^2$.Blank calculated concentrations of Curcumin in calibration standards were determined using the best fit regression curve calculated. The calibration curve was shown to be linear for Curcumin as shown. The correlation coefficients (r^2) were consistently greater than 0.99 during the course of method validation.

Carry Over Test

Carry over test is used for the calculating the percentage peak area obtained in a processed blank. Plasma injected in duplicate immediately after a processed ULOQ calibration standard which were used from PA batch sample. No significant carry over observed for Curcumin and its internal standard. The carry over test results **(shown in Table 4)**.

Signal-to-Noise (S/N) Ratio

The signal-to-noise ratios were determined for Curcumin at LLOQ concentrations in nine independent lots of K_2EDTA.Human normal plasma including haemolysed plasma, one lot of lipemic plasma and one lot of heparin plasma. Signal-to-Noise ratios ranged from 86.344 to 226.594 across the matrix lots evaluated, demonstrating acceptable S/N intensity

RECOVERY

The recovery of Curcumin was determined by comparing the detector response of curcumin at three distinct levels of extracted low, medium and high-quality control samples from extracted Calibration curve and quality control samples from PA batch with detector response obtained from un-extracted aqueous quality control samples. The average recovery of Curcumin was 66.72%. The percentage CV for Curcumin was 3.91% at three different QC level **(Shown in Table 5)**.

Recovery of Internal Standard

The recovery of Internal standard was determined by the average detector response of internal standard in extracted low, medium and high-quality control samples from extracted Calibration curve and quality control samples from PA batch with average detector response obtained from un-extracted aqueous quality control samples. The mean recovery of internal standard was 83.04 % for Curcumin D6.

The recovery of internal standard **(shown in Table 6)**.

K_2EDTA Plasma Screening

Selectivity was evaluated by analysing a total of nine lots (six lots of blank K_2EDTA human normal plasma, one lot of haemolysed plasma, one lot of lipemic plasma and one lot of heparin plasma), obtained from independent sources No significant interferences were observed at the retention times of analyte and internal standard in nine out of nine lots evaluated, demonstrating acceptance criteria were met **(Shown in Table 7)**.

The representative chromatogram of standard blank for curcumin is **(shown in Figure 2)**, the representative chromatogram of standard curcumin **(shown in Figure 3)** and the calibration curve of curcumin is **(Shown in Figure 4)**.

Table 2: Curcumin stock dilution for calibration curve

STOCK	STOCK COCENTRATION [µg/ml]	STOCK ALIQUOT [ml]	DILUENT ADDED [ml]	FINAL VOLUME	FINAL CONC [µg/ml]	STOCK CC
CURC	1004.2	0.049	0.951	1	49.2	STD H
STD H	49.2	0.755	0.245	1	37.1	STDG
STD G	37.15	0.5	0.5	1	18.5	STD F
STD F	18.57	0.5	0.5	1	9.28	STD E
STD E	9.28	0.5	0.5	1	4.64	STD D
STD D	4.64	0.5	0.5	1	2.32	STD C
STD C	2.32	0.3	0.7	1	0.696	STD B
STD B	0.69	0.36	0.64	1	0.25	STD A

Table 3: Precision and accuracy batch for curcumin

Standard	Units	A	B	C	D	E	F	G	H	SLOPE	INTERCEPT	r^2
Actual Concentration	(ng/mL)	0.418	1.126	3.41	6.818	13.636	27.27	54.538	72.236			
Calculated Concentration	(ng/mL)	0.4	1	3.2	7.1	14	27.5	55.8	72.8	0.4576	0.0241	0.9977
%Nominal		95.69	88.81	93.84	104.1	102.67	100.8	102.31	100.78			

QC ID	LQC	INTQC	MQC	HQC
Actual Concentration (ng/mL)	1.124	6.813	27.251	54.177
Calculated Concentrations (ng/mL)	*1.9000	7.1	27.4	55.3
	1.2	7.1	28.7	55.6
	1.1	6.9	27.6	55.8
	1.1	7.1	28.4	54.2
	1.1	7.4	27.5	54.1
	1.1	6.9	27.7	55.7
Mean	1.12	7.08333	27.88333	55.11667
SD	0.044721	0.183485	0.534478	0.767898

Table 4: Carry over test

Sample ID	Analyte Peak Area	IS Peak Area
Extracted Blank	50	15
Extracted LLOQ+IS	2621	11302
Extracted ULOQ+IS	286776	9235
Extracted Blank –I	269	3
Extracted Blank-II	0	10
Average of Extracted Blank-I & II	8.36	-0.12
% Carry Over	-1.91	-0.05

Table 5: Recovery ofcurcumin

Quality Control Samples ID	Aqueous Analyte Area	Extracted Analyte Area
LQC	12761	*13025
	11268	7739
	10805	7862
	10585	6889
	10658	7625
	9937	7044
Mean	11002	7432
SD	962	436
%CV	8.75	5.87
% Recovery	67.55	
MQC	277581	161687
	294032	182603
	260466	167733
	253315	164173
	271483	186797
	251931	163497
Mean	268135	171082
SD	16199	10812
%CV	6.04	6.32
% Recovery	63.8	
HQC	536483	335540
	513342	348466
	509996	351366
	503572	370669
	518734	360691
	536896	379863
Mean	519837	357766
SD	13949	16030
%CV	2.68	4.48
% Recovery	68.82	
Recovery Result		
LQC	67.55	
MQC	63.8	
HQC	68.82	
MEAN	66.72	
SD	2.61	
%CV	3.91	

Table 6: The recovery of internal standard curcumin D6

Quality Control Samples ID	Aqueous IS Area	Extracted IS Area
LQC	16283	14667
	16796	13469
	15923	14616
	15723	13626
	15684	14534
	15267	13891
MQC	15934	12871
	16236	13875
	15566	13238
	15374	12621
	15775	14790
	15464	12894
HQC	18876	13256
	18278	13688
	18553	13741
	18188	14930
	18540	14566
	18781	14875
Mean	16735.61111	13897.1111
% Recovery	83.04	

Table 7: K_2EDTA plasma screening

Plasma lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio (≥5)
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS (<5%)	Analyte/IS	Analyte
MAT-0558	13	10	4938	26400	0.2633	0.0379	0.187	226.594
MAT-0559	15	26	4348	25772	0.345	0.1009	0.169	176.574
MAT-0560	1	18	4621	26743	0.0216	0.0673	0.173	86.344
MAT-0568	48	0	4324	25538	1.1101	0	0.169	159.454
MAT-0569	28	1	4817	27043	0.5813	0.0037	0.178	151.499
MAT-0570	0	3	4406	25862	0	0.0116	0.17	147.361
MAT-0617 (Heparin)	0	34	4677	26714	0	0.1273	0.175	140.671
MAT-0515 (Hemolysed)	11	21	4931	26146	0.2231	0.0803	0.189	133
MAT-0510 (Lipemic)	18	69	4523	26263	0.398	0.2627	0.172	163.89
				Mean	26275.66667	0.32692	0.08173	0.17578
							SD	0.00753
							%CV	4.28

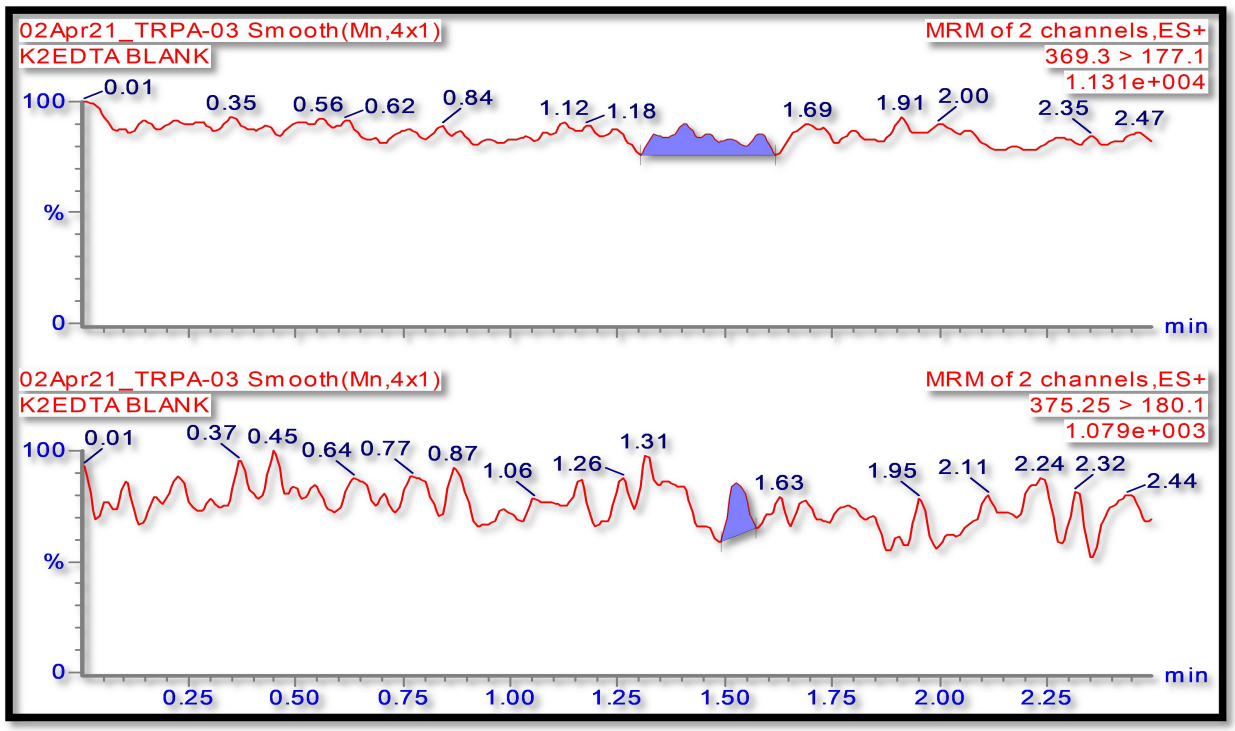

Figure 2: Representative chromatogram of a standard blank for curcumin

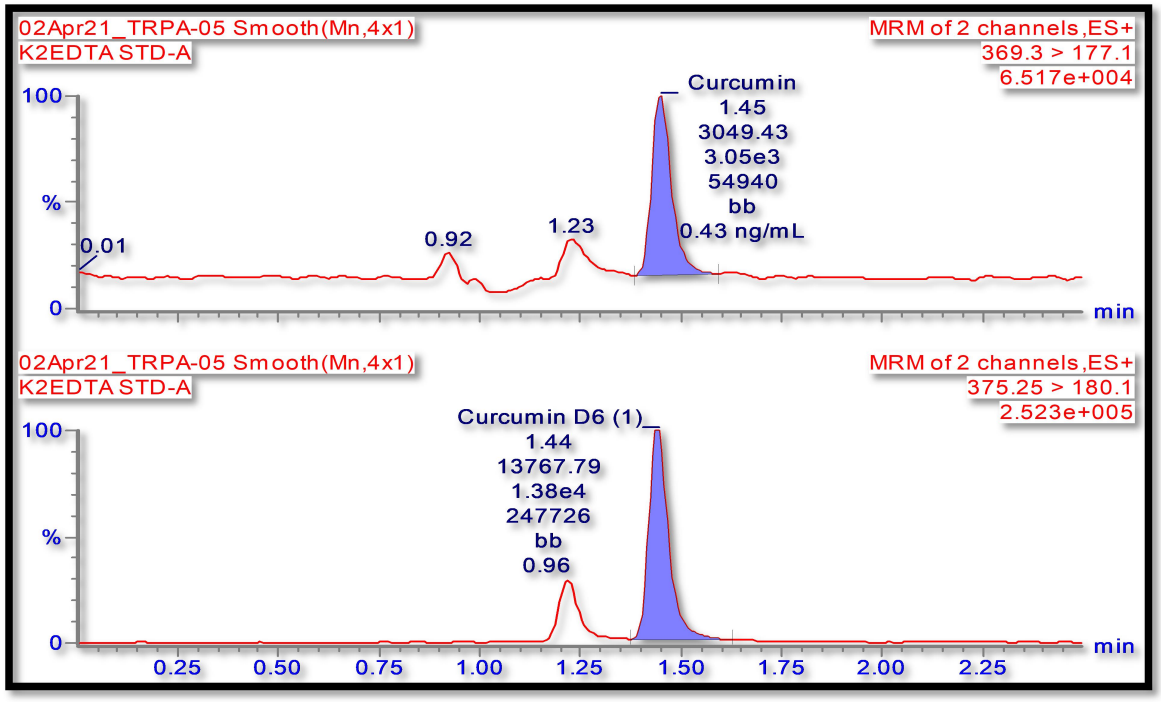

Figure 3: Representative chromatogram of a standard a for curcumin

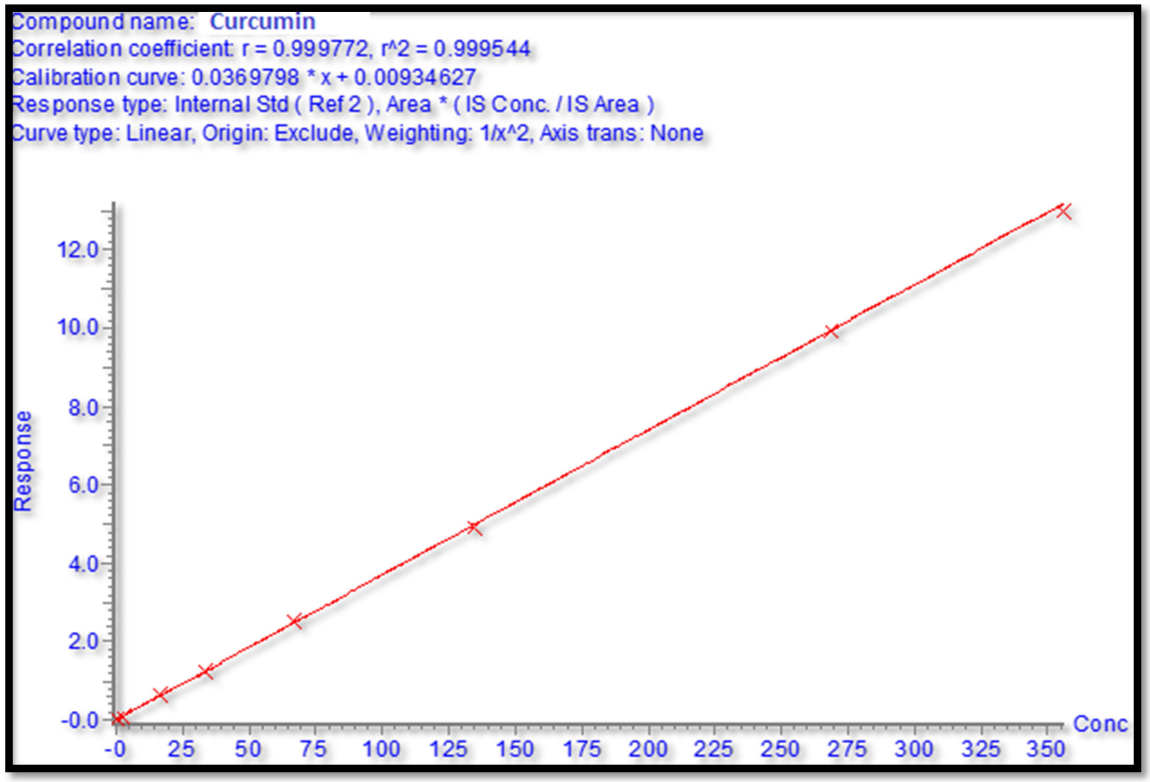

Figure 4: Calibration curve of curcumin

CONCLUSION

The Bio analytical methods are accurate and reliable to determine these drugs at lower concentrations. Selectively Liquid Chromatography-Mass Spectrometry method to quantitate Curcumin in K_2EDTA.Human plasma over the concentration range 0.4180 to 72.2360 ng/mL was successfully validated. This method is suitable for sample analysis to support bio-equivalence/bioavailability studies involving formulations of Curcumin. By ultra-performance Liquid chromatography UPLC–MS/MS is currently considered as the best choice. Curcumin is quantified through UPLC-MS/MS studiesand it has high specificity, moresensitivity, andincreased rapidity in analysis.

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