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Research Article

Determination of Curcumin Sulphate in Human Plasma by High Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS/MS)

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ABSTRACT

A simple and sensitive method for the determination of curcumin sulphate, in human plasma by High Performance Liquid Chromatography coupled with mass spectrometry method has been developed and validated. Nevirapine is used as an internal standard. Analyte and internal standard were extracted with ethyl acetate and isopropyl alcohol from human plasma, separated on a Zorbax Eclipse plus c^{18} 150, 4.6mm, 5µm column. An AB MDS SCIEX 4000 Q trap mass spectrometer was used under multiple reaction monitoring mode (MRM) for quantification of transitions at m/z 447.20, 134.00 and 265.10, 182.00 under negative ion mode for curcumin sulphate and nevirapine respectively. The intra and inter batch precision and accuracy studies were well within the acceptance limits. The linearity of curcumin sulphate was shown in the range of 25-10000.00ng/ml with 85-115% accuracy and < 20% precision in human plasma. The method has been proved to be simple, sensitive, fast, reliable, rugged and reproducible. The validated method can be applied for the estimation of curcumin sulphate in plasma samples for pharmacokinetic studies.

Keywords: Curcumin sulphate; Nevirapine; HPLC-MS/MS; Validation; Human plasma.

1. INTRODUCTION

Curcumin (1,7-bis (4-hydroxy-3-methoxy phenyl)-1,6-hepta-diene-3,5-dione is a phenolic compound derived from the spice herb Curcuma longa L., widely used in food industry as a natural colouring agent and curry powder[1]. It is an active ingredient of turmeric. Turmeric compounds are classified into two important groups of natural products, the diaryl heptanoids and sesquiterpinoids which are responsible for producing many biological and medicinal activities[2]. The curcuminoids including curcumin, de-methoxy curcumin, bis-demethoxy curcumin were the major diaryl heptanoids[3]. Curcuminoids are recognized for their broad spectrum of biological activity and safety in foods (or) pharmaceuticals[4]. Use of curcumin as a folk remedy continuous today[5,6]. Oral administration of curcumin metabolized through biotransformation resulting in curcumin glucuronide and curcumin sulphate[7,8]. Metabolic studies have demonstrated that orally administered curcumin is extensively transformed to curcumin-o-glucuronide and curcumin-o-sulphate both in rodents and humans[9-11]. Low levels of curcumin in human biomatrices and extensive metabolism following oral dosing suggest that further clinical development of curcumin would benefit from highly sensitive analytical methods for the analysis of curcumin metabolites. Curcuminoids determination in plasma by HPLC-MS/MS has been reported by several workers[8,15-22]. The structure of curcumin sulphate and nevirapine was given in fig 1.

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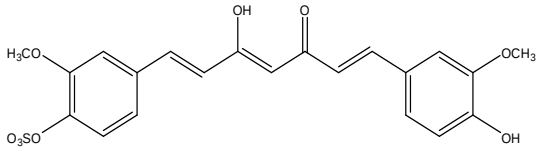

(a) Structure of curcumin sulphate

(b) Structure of Nevirapine{IUPAC name:11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido-(3,2-b,2^1,3^1-e) (1,4) diazepine-6-one.}

Fig.1: Chemical structure of curcumin sulphate and nevirapine

Here we report the development and validation of a novel HPLC-MS/MS method for the quantification of curcumin sulphate, a metabolite of curcumin, using nevirapine as an internal standard, by using negative ion mass spectrometry. The results showed that the HPLC-MS/MS is a powerful tool to study the invitro metabolites of curcumin, allowing the identification of very low level (ng/ml).

2. MATERIALS AND METHODS

2.1 Plasma samples

K_2EDTA plasma was collected from commercially procured human whole blood. Plasma blank from six donars was chromatographically determined for interfering substances prior to use.

2.2 Chemicals and reagents

Curcumin sulphate working standard (M.wt. 448.44, purity 99.76) was procured from sigma Aldrich, and Nevirapine anhydrous as an internal standard (M. wt.266.298, purity 99.9%) was procured from sigma Aldrich. Acetonitrile (HPLC grade/equivalent), ammonium acetate (GR grade/equivalent), K_2EDTA human plasma, dimethyl sulfoxide (GR grade),methanol (HPLC grade/equivalent),water (HPLC grade/equivalent), acetic acid (GR grade/equivalent), ethyl acetate (GR grade/Equivalent), iso propyl alohol (GR grade/equivalent) were used.

2.3 Preparation of standard solutions

Primary stock solution of curcumin sulphate was prepared in 10% DMSO in methanol and stored at 2-8^{0}C. From the stock solution appropriate dilutions of curcumin sulphate was made in the range of 0.500µg/ml to 200.000µg/ml with 60% acetonitrile in water for calibration curve standards. From the above dilutions of calibration curve standards, the spiked K_2EDTA plasma samples for calibration curve standards were prepared, the curcumin sulphate in the concentration range of 25.000ng/ml to 10000.000ng/ml and labeled as CC_1 to CC_8 (25.000ng/ml, 50.000ng/ml, 250.000ng/ml, 500.000ng/ml, 1500.000ng/ml, 3000.000ng/ml, 6000.000ng/ml, 10000.000ng/ml) .

Another set of working solutions of curcumin sulphate in the concentration range of 0.500µg/ml to 340.00µg/ml were prepared from stock solution by appropriate dilutions. From the working standards of curcumin sulphate, spiked K_2EDTA human plasma for QC were prepared in the concentration range of 25.000ng/ml to 17,000.000ng/ml and labeled as, lower limit of quantitation (LLOQ-QC), low concentration (LQC), geometric mean concentration (GMQC), medium concentration (MQC), high concentration (HQC) and dilution integrity quality control samples (DIQC) respectively. (25.000ng/ml, 50.000ng/ml, 550.00ng/ml, 4000.00ng/ml, 8000.000ng/ml, 17,000ng/ml). From the QC standards of the curcumin sulphate, 75.000ng/ml and 8000.000ng/ml concentration of curcumin sulphate with hemolytic and lipemic plasma were prepared and labeled them as hemolytic LQC, hemolytic HQC and lipemic LQC and lipemic HQC respectively. The primary stock solution of an internal standard (nevirapine) was prepared, and working standard, 2000.000ng/ml was prepared by dilution. These solutions were stored at 2-8 ^{0}C until the completion of analysis.

2.4 Sample processing

The blank, calibration curve standards, quality control samples from the deep freezer were withdrawn and allowed to thaw. Vortexed the thawed samples to ensure complete mixing of the contents. 20µl of 60% acetonitrile in water solution was transferred to a ria vial which was labelled as plasma blank. 20µl of internal standard was added to the prelabelled ria vials (except plasma blank), then transferred the 0.200ml of plasma

to the above vials from the respective samples and vortexed the samples to ensure complete mixing of contents. 0.200ml of 0.1% acetic acid in 10mM ammonium acetate was added to all the samples and vortexed to ensure complete mixing of contents. To this 2.5ml of extraction solution (a mixture of 700.00ml of Ethyl acetate and 300.00ml of iso propyl alcohol was prepared and sonicated) was added and placed on a shaker for 10 minutes and centrifused for 10 minutes at 4000 rpm at 20^0C and transferred the supernatant (organic layer) into another prelabelled ria vial. Evaporated this layer under a stream of nitrogen gas at 45 ^{0}C. The residue was reconstituted with 0.200ml of reconstitution solution (10% DMSO in methanol) and vortexed. The sample was loaded into the auto-injector vials and 10µl of sample was injected onto the LC-MS/MS system.

2.5 Instrumentation and Chromatography

A HPLC system (Agilent 1200 series), equipped with MS (AB MDS SCIEX 4000 Qtrap) consisting of Zorbax Eclipse plus C^{18} 150, 4.6mm, 5µm column was used for validation. The column oven temperature was set at 40^0C. The profile of the gradient elution was: (A) 0.1% acetic acid 10mM Ammonium acetate and (B) Acetonitrile, 0-4.50 min, 25-75% B; 4.50-4.51min, 25-75% B, 4.51-9.99min, 60-40% B, 9.00-9.01min, 60-40% B, 9.01-10.00min, 25-75% B at a flow rate of 1ml/min, and the volume of injection was 10µl.

An AB MDS SCIEX 4000Q trap mass spectrometer was coupled with HPLC instrument using a turbo ion spray interface. The turbo ion spray voltage was -4200V and the ion probe temperature was 500^0C. The mass spectra were acquired in the negative ion mode. Multiple reaction monitoring mode (MRM) was used for quantitative analysis of the analytes. At the time of tuning for selection of transitions, the molecule dependant parameters like declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) was optimized by using direct infusion of sample. The spectra were acquired in the negative ion mode. Negative mode (-) turbo ion spray was very effective in identifying diaryl heptanoids from turmeric, because of the presence of phenolic –OH groups, which enables these compounds to be easily ionized in negative ESI mode (23). Quantification was achieved with MRM in negative ion mode for both the analyte and the internal standard. The tuning parameters were curtain gas 20.0psi, turbo ion spray voltage at -4200V, turbo ion spray probe temperature 500^0C, ion source gas 1 at 40.0psi, ion source gas 2 at 45.0psi. Detection of the ions were carried out in MRM, by monitoring the transition of ion pairs at m/z 447.20, 134.00 for curcumin sulphate and 265.10, 182.00 for nevirapine respectively. The analysis data obtained were processed by analyst version 1.4.2 software supplied by the applied biosystems, Canada.

2.6 Method development and validation

The method was validated for selectivity, sensitivity, specificity, matrix effect, linearity, precision, accuracy, dilution integrity, ruggedness and stability. The intra day validation was determined in six replicates at concentrations of 25.000ng/ml, 75.000ng/ml,4000.400ng/ml, 8000.750ng/ml curcumin sulphate. The inter-day validation was determined across these concentrations in triplicates on 3 different days. The calibration curves were fitted by a linear regression with a weighting factor of $1/x^2$. The mean concentrations and co-efficient of variation (CV) of intra-day were calculated as the relative standard deviation (%) from the replicates. The

CV of inter-day was calculated as the relative standard deviation (%) of the respective mean concentrations on each individual day for 3 days. The accuracy of the assay was determined by comparing the corresponding calculated mean concentrations with the nominal concentrations. The results are shown in the table 1 and 2.

Table 1: Intra batch precision and accuracy

S.NO.	Spiked QC concentration ng/ml	Concentration found mean ng/ml	Precision%	Accuracy%
Batch 1	25.000	24.066	3.35	96.21
	74.600	64.634	2.41	86.63
	4000	3985.47	1.86	99.63
	8000.750	7904.17	0.64	98.89
Batch 2	25.000	25.80	5.63	103.23
	74.600	65.77	1.64	88.16
	4000	3963.64	1.95	99.08
	8000.750	7910.70	1.02	98.87
Batch 3	25.000	24.76	1.23	99.07
	74.600	67.99	10.84	91.14
	4000	3931.51	1.58	98.28
	8000.750	7869.57	0.69	98.35

Table 2: Inter batch precision and accuracy for curcumin sulphate (batch 1, batch 2, batch 3 mean)

Spiked QC concentration ng/ml	Concentration found, mean ng/ml	Precision %	Accuracy %
25.000	24.899	4.61	99.59
74.600	66.042	3.73	88.52
4000	3944.749	1.16	98.61
8000.750	7907.690	0.63	98.83

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8^{0}C) was performed, (Auto sampler stability or in-injector stability, Bench top stability and freeze and thaw stability (3 cycles)). The results are discussed in the section 3.2. (table 5)

2.7 Recovery and matrix effects

Matrix effect was investigated to ensure that precision, selectivity and sensitivity were not compromised by the matrix. Matrix effect was performed at two concentrations (LQC and HQC) in eight replicates each for analyte along with the internal standard. One set of eight different blank matrix samples used for spiking the LQC concentration and internal standard, and another set of eight different blank matrix samples for spiking the HQC concentrations and internal standard were analysed. The samples prepared equivalent to LQC and HQC concentrations by adding analyte and internal standard to reconstitution solution and perform the analysis by injecting 6 replicates each.

Hemolytic and lipemic matrix effect were determined by using 6 samples at each of LOQ and HQC samples of analyte concentration, which were spiked in hemolytic and lipeic plasma separately. The samples were analysed under a calibration curve. For hemolytic plasma at LQC, HQC the mean % accuracy was 98.008, 96.81 and CV% was 2.27, 4.31 and for lipemic plasma at LQC, HQC the mean % accuracy was 89.13, 103.94 and CV% was 4.074, 2.67 respectively. The internal standard normalized factor as calculated by the CV% is less than 15%.

The recovery experiments were performed in six replicates for curcumin sulphate along with the internal standard by comparing the analytical results for extracted samples at 3 concentrations LQC, MQC and HQC with unextracted sampes that represent 100% recovery. The % of recovery of curcumin sulphate and internal standard was calculated by the formula,

$$\% \text{ Recovery} = \frac{\text{mean response of extracted sample}}{\text{mean response of unextracted sample}} \text{x100}$$

The average % of recovery of the curcumin sulphate was 70.63 % and that of the internal standard was found to be 69.79%.

2.8 Dilution integrity

The dilution integrity was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations of above the ULOQ during real time analysis. Dilution integrity experiment was carried out at 1.7 times the ULOQ concentration of analyte. Six replicates each of dilution factor (DF) 5 concentrations were prepared and their concentrations were calculated by applying the dilution factor 5. The % accuracy and CV% for 1:5 dilutions were found to be 92.53% and 3.012% which are within the acceptance criteria. The results are shown in the table 3.

Table 3: Dilution integrity

Analyte	Dilution factor	DIQC Spiked concentration ng/ml	Concentration found, mean ng/ml	Mean Accuracy %	Precision%
Curcumin sulphate	5	17001.750	15731.579	92.53	3.012

3. RESULTS AND DISCUSSION

Mass spectrometer parameters were tuned in negative ionization mode using turbo ion spray for the analyte and internal standard. For the data acquisition MRM mode was used to get better selectivity. Separation has been achieved by various combination of LC-MS/MS parameters with negative ionization mode. Curtain gas was at 20.000psi, turbo-ion spray voltage was -4200V and ion spray probe temparature was 500^{0}C. Ion source gas 1 at 40.0 psi and ion source gas 2 at 45.0 psi. The retention time of curcumin sulphate and nevirapine was 2.20-3.20, 3.20-4.00. Liquid-liquid extraction technique was employed for the extraction of analyte and internal standard. Liquid-liquid extraction is helpful in producing a spectroscopically clean sample when compared to protein precipitation and avoiding the introduction of plasma components, and non-volatile materials on to the LC-MS

system. An internal standard mimic the analyte during the extraction as well as during the ionization. For HPLC-MS/MS analysis use of Nevirapine as an internal standard, has proved to be helpful, when there is a significant matrix effect.

3.1 Method development and validation

The sensitivity of the method was determined by lower limit of quantification, by analyzing six LLOQ samples in all the three intra and inter precision and accuracy batches. The lower limit of quantification (LLOQ-QC) was found to be 25.000ng/ml for curcumin sulphate, the % accuracy was 99.59% and the mean value of co-efficient of variation CV% was 4.61%.

The selectivity of the present method was established by checking the blank K_2EDTA human plasma, K_2EDTA lipemic and hemolytic plasma (without spiking curcumin sulphate) obtained from eight different donars. (if K_2EDTA human plasma contain any interfering compounds that elute along with curcumin sulphate and internal standard) Six spiked samples at LLOQ concentrations of curcumin sulphate and internal standards in plasma of one of the donar, except hemolytic and lipemic plasma. The response of analyte and internal standard were compared with the mean response of LLOQ concentration, there were no significant interfering peaks found at curcumin sulphate retention time in the plasma blanks. Fig. 2, 3, shows the representative chromatograms of the blank K_2EDTA human plasma samples. The representative chromatograms at different concentrations, LLOQ, LQC, MQC, HQC levels are shown in figs. 4, 5, 6, 7. The specificity of the present method was established by checking the interferences at curcumin sulphate retention time caused due to internal standard by injecting six replicates of MQC concentration of curcumin suphate. The response of analyte and internal standard were compared with the mean response of LLOQ concentration injected. The results showed that there were no significant interfering peaks obtained at curcumin sulphate retention time due to internal standard and at nevirapine retention time caused due to curcumin sulphate.

The precision and accuracy of the method was evaluated by the CV% and accuracy % at different concentration levels corresponding to LLOQ, LQC, MQC, HQC, during the course of validation. The precision and accuracy of analyte in intra and inter batch results were within ±15%, and results are shown in the table 1 and 2. (in section 2.6, method development and validation). Linearity was determined by using $1/x^2$ for curcumin sulphate and 1/x for nevirapine by weighted least square regression analysis by the standard plots associated with a eight point standard curve. All the calibration curves were analysed during the course of validation. The method was found to be linear over the concentration range of 25-10000ng/ml for curcumin sulphate. The concentration of unknown sample was calculated from the equation by using regression analysis of spiked plasma calibration standards with $1/x^2$ as weighting factor.

$$Y = mx + c$$

Where,

Y = ratio of curcumin sulphate peak area and nevirapine peak area. (analyte area/internal standard area), x = concentration of curcumin sulphate, m = slope of the calibration curve, c = y-axis intercept value.

Ruggedness was performed by using a different lot of the same column manufactures and different analyst. The precision and accuracy for the quality control samples at HQC, GMQC, MQC and LQC concentration levels were found to be with in the acceptance limit of 15% , for all the samples of LLOQ QC was found to be with in the acceptance limit of 20%, and the results were showed in the table no. 4a, 4b. (4a-for different column, 4b-for different analyst)

Table 4a: Ruggedness: precision and accuracy for curcumin sulphate by different columns

Spiked QC concentration ng/ml	Concentration found, mean ng/ml	Precision%	Accuracy%
25.000	24.056	3.35	96.21
74.600	64.634	2.41	86.63
4000	3985.47	1.86	99.63
8000.750	7904.176	0.64	98.89

Table 4b: Ruggedness: precesion and accuracy for Curcumin sulphate by different analysts

Spiked QC concentration ng/ml	Concentration found, mean ng/ml	Precision%	Accuracy%
25.000	25.808	5.63	103.23
74.600	65.771	1.64	88.16
4000	3963.64	1.95	99.08
8000.750	7910.700	1.02	98.87

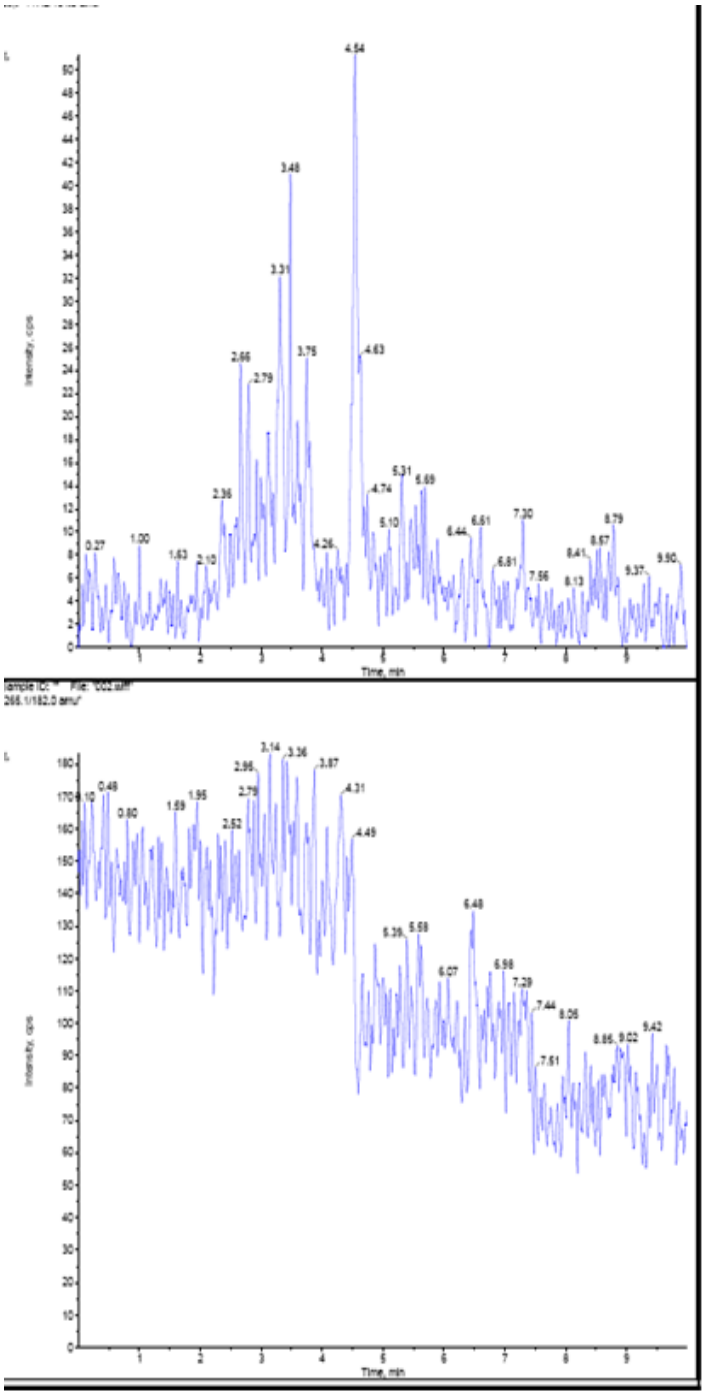

Fig. 2: Selectivity blank plasma

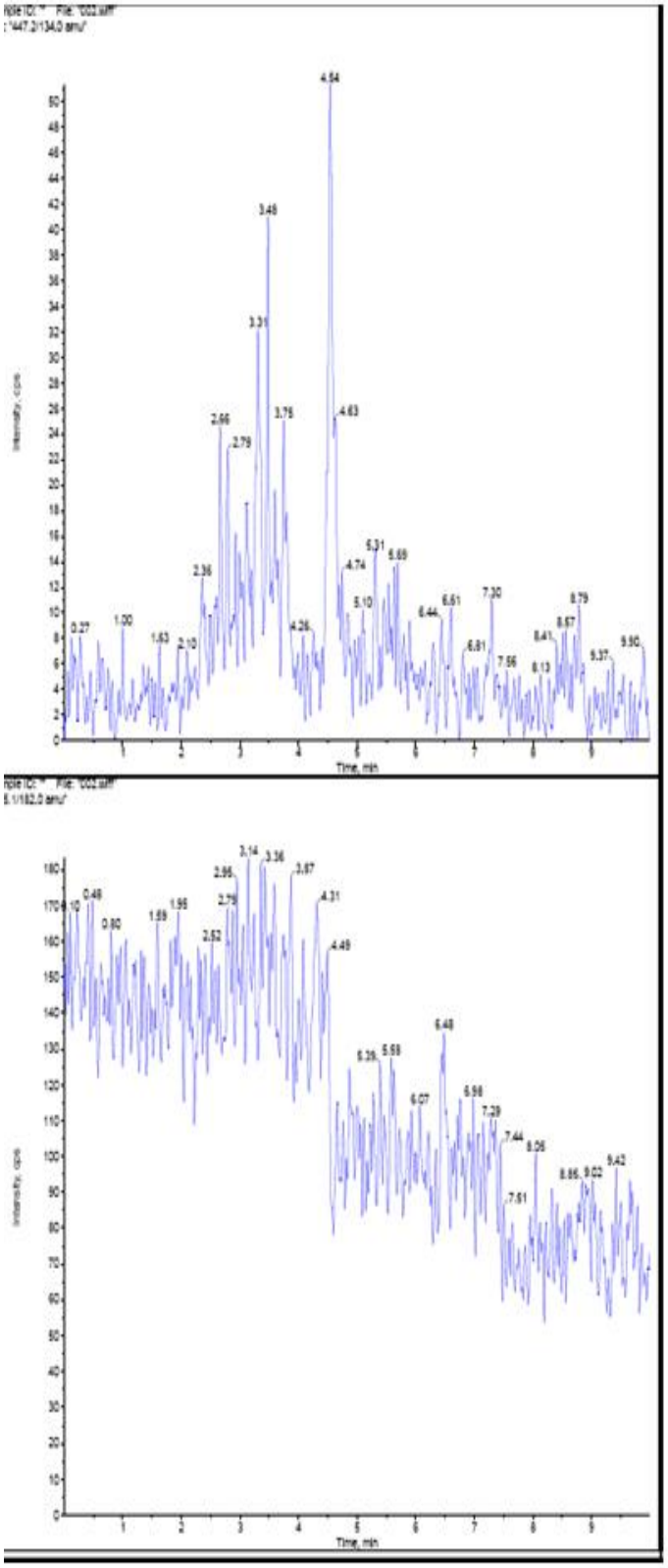

Fig. 3: Precision and accuracy blank plasma

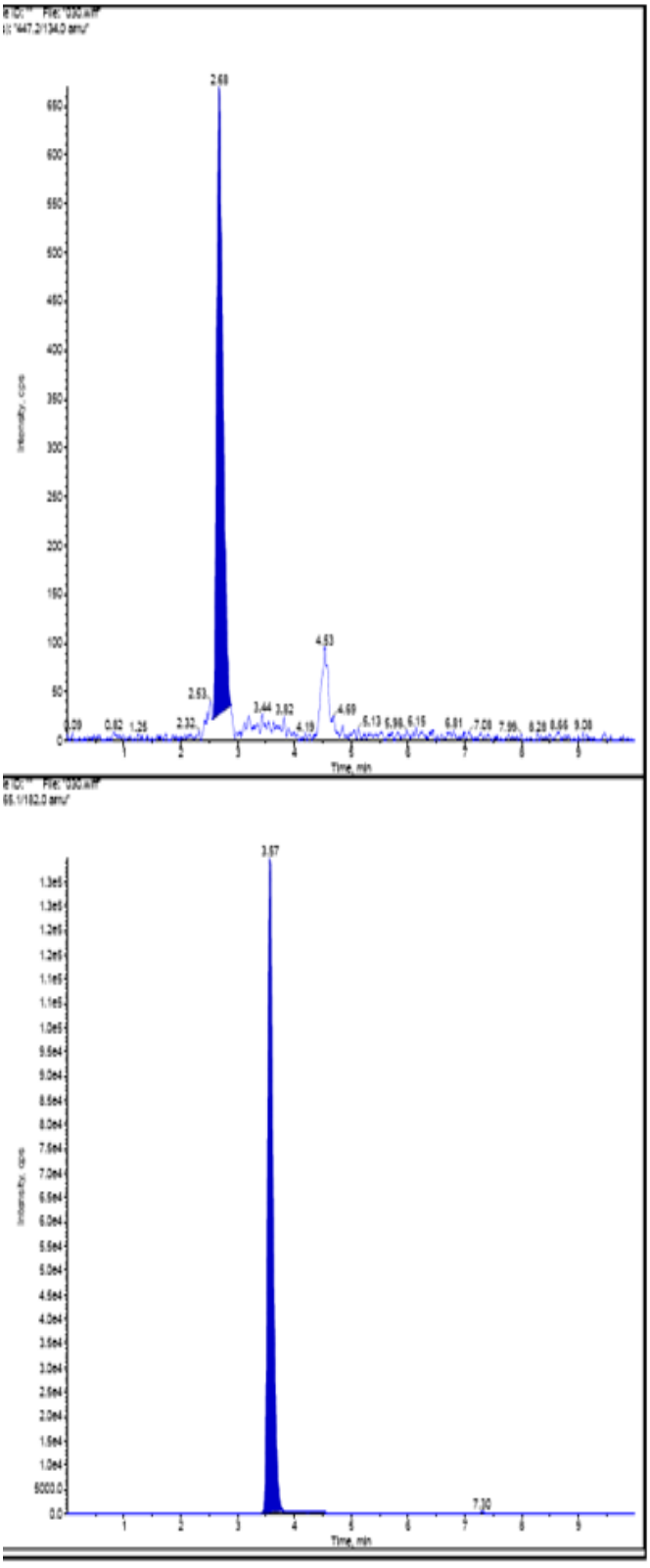

Fig.4: Precision and accuracy-LLOQ

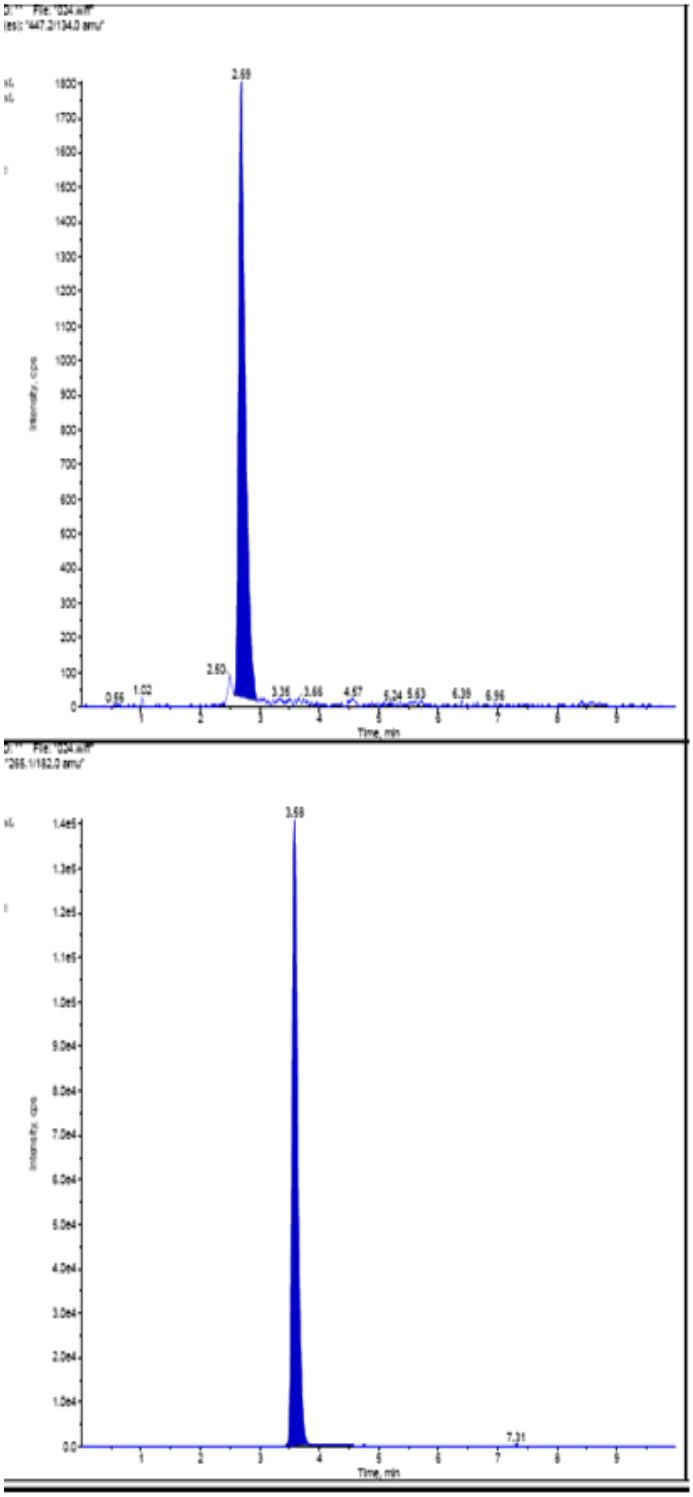

Fig. 5: Precision and accuracy-LQC

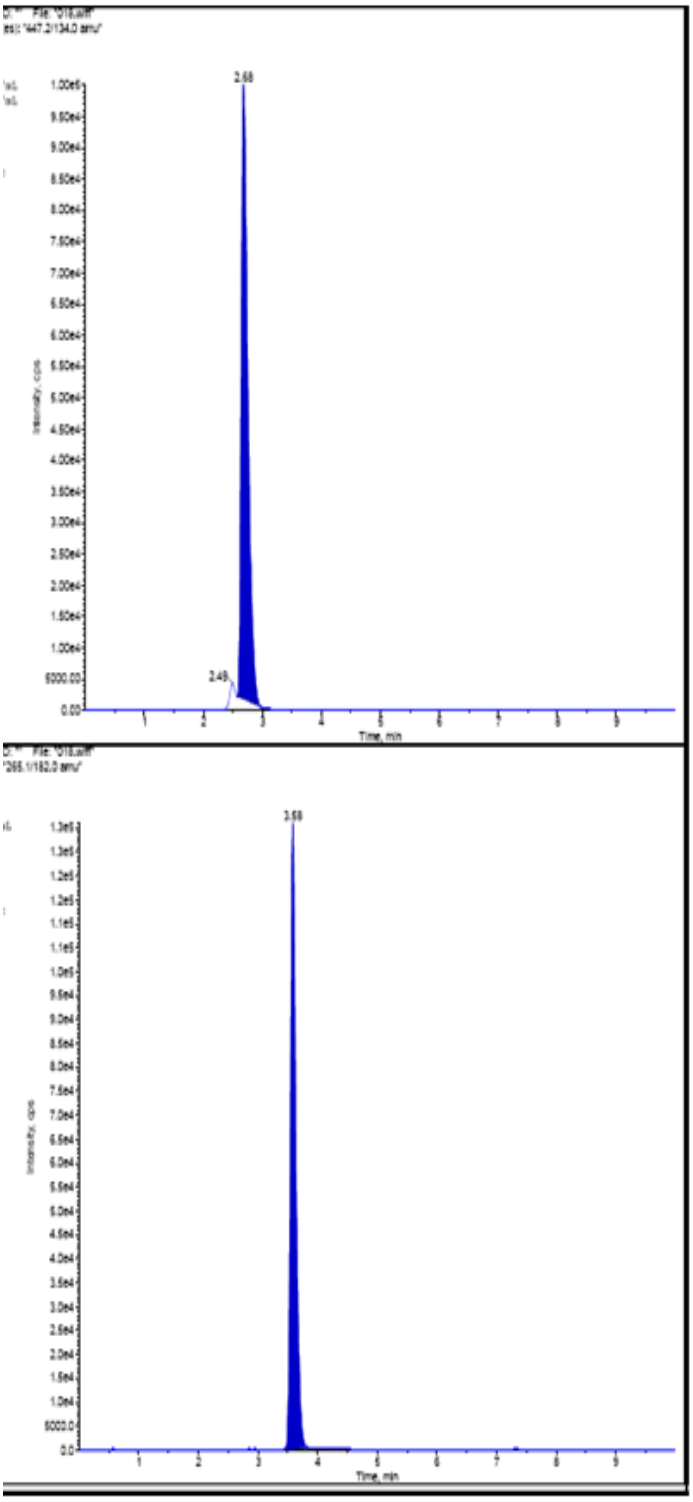

Fig. 6: Precesion and accuracy-MQC

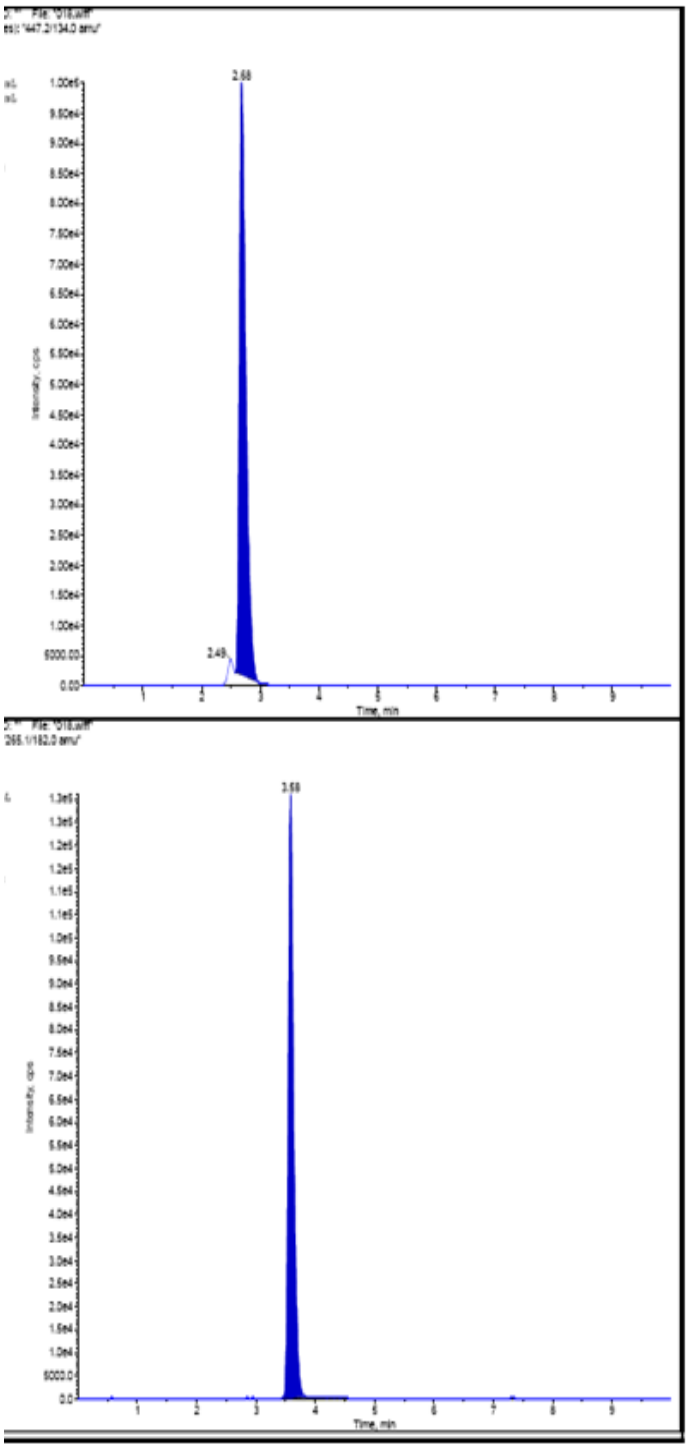

Fig. 7: Precision and accuracy-HQC

3.2. Stability of Curcumin sulphate in human plasma

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8^{0}C) was performed by comparing the area response of the analyte with the response of the sample prepared from fresh stock solution. Auto sampler or in-injector stability (54hours, bench top stability (27.00 hours), and freeze and thaw stability (3-cycles), long-term stability test (200days) were performed at low and high QC levels using 6 replicates at each level and stability of analyte in plasma has been proved at room temperature (10 hours) and refrigerated temperature (18.00hours). The mean % nominal values of the analyte were found to be within 15% of the predicted concentrations of the analyte at their low and high QC levels. The results showed within the acceptance limit during the entire validation. The results are summarized in the table 5.

Table 5: Stability of curcumin sulphate in human plasma under different conditions

Stability test	QC spiked concentration ng/ml	Mean ng/ml	Accuracy or stability (%)	Precision (%)
[a]Auto-sampler or in-injector	74.600	76.110	102.022	4.19
	8000.750	7334.050	91.785	3.232
[b]Freeze and thaw	74.600	72.933	97.761	3.367
	8000.750	7761.500	97.005	7.691
[c]Bench top	74.600	68.226	91.45	4.85
	8000.750	7848.133	98.084	3.785
[d]Long-term	74.600	79.7541	106.903	2.345
	8000.750	7995.873	99.935	3.519

([a] 54 hours in auto sampler at 10 ^{0}C, [b] after 3 freeze and thaw cycles, [c] 27 hours on bench, [d]long-term stability -200 days)

The sample preparation technique, described offers a rapid measure of processing samples for the assay of total curcumin sulphate. Thus making the assay simple, rapid and rugged. It is a simple HPLC method for the analysis of curcumin sulphate in plasma. The method enables to carryout highly accurate analysis, thus making it sensitive, specific and selective in the range 25-10000ng/ml.

4. CONCLUSION

A simple, rapid and specific HPLC-MS/MS method was developed and validated for quantification of curcumin sulphate in human plasma. This method provides a comprehensive analytical method to characterize the pharmacokinetics of curcumin sulphate, in human plasma and thus, it can be used as an analytical tool for curcumin metabolite studies.

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