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ANALYSIS OF CELECOXIB IN HUMAN PLASMA WITH MEASUREMENT UNCERTAINTY ESTIMATION BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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ABSTRACT

This paper describes a simple and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification of celecoxib in human plasma using celecoxib d7 as internal standard (IS). A Zodiac C_{18} (50×4.6mm) 3.0µm column with isocratic mobile phase of 5mM ammonium formate buffer, acetonitrile and methanol (20:20:60,%v/v/v) was used at a flow rate of 1 mL/min. The proposed linearity for celecoxib was 5.047-2518.667 ng/mL. A total of five linearity curves were generated with quality control sample to calculate the precision and accuracy. Also the stability of analyte was extensively evaluated in plasma as well as in extracted samples and results met the acceptance criteria defined in USFDA guidelines. The chromatographic run time was set at 2.5 min which makes the proposed method high throughput. Intra- and inter-day precision defined by coefficient of variation was <10% and accuracy (bias %) was within 90–110%. Measurement uncertainty estimation was 11.2% for Celecoxib. The method has been successfully used for the analysis of celecoxib in human plasma.

KEYWORDS: Celecoxib, Solid phase extraction, Method validation, LC-MS/MS, Measurement Uncertainty.

INTRODUCTION

Celecoxib is a COX-2 selective nonsteroidal anti-inflammatory drug (NSAID). It is used to treat the pain and inflammation of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute pain in adults, painful menstruation, and juvenile rheumatoid arthritis in people two years or older. After oral administration, Celecoxib is rapidly absorbed and achieves peak plasma concentration in about 3hours. It is extensively metabolized in the liver and being eliminated little exchange (3%). The major routes of excretion for Celecoxib are feces and urine. Many analytical methods based on LC-MS/MS,[1-5] HPLC, UV methods were reported for the determination of celecoxib in human plasma samples. Satheeshmanikandan R, et.al were reported simultaneous determination of celecoxib, erlotinib, and its metabolite desmethyl-erlotinib (OSI-420) in rat plasma by LC-MS with LOQ of 1.5 ng/mL for in vivo studies.[6] David S Alberts, et.al reported celecoxib in human plasma using solid-phase extraction by high-performance liquid chromatography with LOQ of 40 ng/mL.[7] Hassan Jalalizadeh, et.al reported celecoxib in human plasma by high-performance liquid chromatography with LOQ of 10 ng/mL using liquid–liquid extraction with chloroform,[8] Some other HPLC methods also reported by Zarghi, et. al,[9] Elke Stormer, et. Al,[10] Gugulothu P, et al,[11] Jadhav AS, et.al,[12] Brutigam L, et. al[13] have some critical steps which are not very effective to perform. Abdel-Hamid, et. Al,[14] were reported Liquid chromatographic–mass spectrometric determination of celecoxib in plasma using single-ion monitoring in a run time of 4 min. Ulrike Werner, et. al[15] were reported LC-MS by chemical ionization (APCI) liquid–liquid extraction but with an LOQ of 5 µg/L. To overcome all the drawbacks and disadvantages of existing methods, we have developed and validated a simple, more reliable and rapid LC-ESI-MS method for the determination celecoxib in human plasma by solid phase extraction technique in the range of 5-2500 ng/mL with a run time of 2.5 minutes only. Reliable analytical results are needed for correct interpretation of quantitative findings in biopharmaceutics. For this reason, it is important to provide information on uncertainties of their analytical results. Uncertainty of any measurement is the doubt which exists about the result of a test sample, and it represents a quantitative value of the doubt about the measurement result. Thus, knowledge of the uncertainty is required for correct interpretation of the measurement because such a result is only complete if it is accompanied by a statement of uncertainty in the measurement. Recently, Lee et al.[16] reported on measurement uncertainty (MU) estimation by the bottom-up approach for amphetamine and methamphetamine in urine as a validation data. Measurement of uncertainty estimation is included in this analytical result to re-emphasize the importance of MU estimation of such drugs which effect the livelihood. In this study, a sensitive, rapid, and defensible method by LC–MS was developed with information on MU estimation.

EXPERIMENTAL

Materials

Celecoxib was supplied by Sigma-aldrich, India. Acetonitrile and methanol of MS grade and all other chemicals of analytical grade were purchased from Merck, India. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore. Drug-free plasma containing sodium citrate anticoagulant was kindly donated by human healthy volunteers and stored at -23°C until use.

Instrumentation

The LC–MS/MS analysis was carried out in turbo ion spray ionization in negative mode on a mass spectrometer (API 4000) coupled to a Shimadzu LC system (Model: SIL-HTC) operated with Analyst 1.6.1 software.

Chromatographic and MS parameters

The separation of the analytes was carried out on an Zodiac C18 (50 mm length x 4.6 mm internal diameter and 3.0 µm particle size) column. Temperature was set to 35°C. The mobile phase composed of 5mM ammonium formate, acetonitrile and methanol (20:20:60%v/v) at a flow rate of 1.0 mL/min (with splitter) for a runtime of 2.5 min. The injection volume was set at 15 µL. The full scan MS and MS/MS spectra of analyte was obtained by direct infusion of the respective sample solution at a concentration of 10 µg/mL solution prepared in methanol. The samples were analyzed using multiple reaction monitoring (MRM) mode. Optimization of the signal was performed by constant injection of high concentration of celecoxib and ISTD. The transition of the parent to product ion was studied with the use of a turbo ion spray ionization source, operating the negative ionization mode. The transition of parent to production (m/z) for celecoxib was 380 (parent) and 316 (product). Dwell time (milli sec), declustering potential (DP), collision energy (CE) and collision exist potential (CXP) were optimized at 200 ms, 101, 31 and 13 volts, respectively. The parent to product ion (m/z) for ISTD was 387.20→ 323.20.

Preparation of calibration samples and quality control samples

Stock solutions

The stock solution was prepared by dissolving 2.0320 mg of celecoxib working standard in 2 mL methanol to a final concentration of 1.00 mg/mL. The internal standard stock solution was prepared by dissolving 2.0186 mg of celecoxib d7 in 2 mL HPLC grade methanol to a final concentration of 1.00 mg/mL.

Working standard solutions

The stock dilutions of celecoxib was prepared in the concentrations ranging from 100.200 to 50000.00 ng/mL with the diluent for calibration curve standards.

Quality control (QC) samples

0.250 mL of above stock dilutions ranging from 100.200 to 50000.000 ng/mL were transferred to the pre-labelled volumetric flasks and made up the volume to 5 mL with human plasma.

Sample Preparation

The samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. 200 µL of the plasma sample was pipetted into pre-labelled RIA vial tubes, 20 µL of internal standard dilution (15.000 ng/mL of Celecoxib d7) was added to it and vortexed, except in blank plasma samples where 20 µL diluent was added and vortexed. Then, 625 µL of 5 mM ammonium formate buffer in 0.1% formic acid was added and vortexed. The sample mixture was loaded onto Celerity Deluxy, (OROCHEM) DVB-LP (30 mg/1mL) cartridges that were pre-conditioned with 1.0 mL of HPLC grade methanol followed by 1.0 mL HPLC grade water (New cartridge for each sample). After applying the maximum pressure the extraction cartridge will be washed with 2.0 mL of HPLC grade water (each time 1.0 mL) followed to 1.0 mL washing solution. Then, the sample will be eluted with 1.0 mL of mobile phase and transferred into vials and loaded into auto sampler.

Method Validation

The LC-MS/MS method was fully validated according to the Food and Drug Administration (FDA) Bio analytical Method Validation guidelines. The entire method was validated for Carry over test, selectivity, sensitivity, matrix effect, Linearity, Precision and accuracy, recovery, dilution integrity, ruggedness, stabilities, concomitant drug effect, reinjection stability, stability in whole blood.

Linearity

Ten concentration levels were prepared in the biological matrix by spiking with known concentration of analyte and constructed the calibration curves (CC). A regression equation with a weighting factor of 1/ (concentration ratio)^2 of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Celecoxib in human plasma. Correlation coefficient (r^2) was greater than 0.99 in the concentration range of 5.047 ng/mL to 2518.667 ng/mL for Celecoxib. The results are represented in Table1.

Table1: Linearity Data for Celecoxib.

Celecoxib CC#	Slope	Intercept	R	r^2
1	0.0007	-0.0006	0.9972	**0.9944**
2	0.0007	-0.0006	0.9984	**0.9968**
3	0.0007	-0.0005	0.9989	**0.9978**
4	0.0007	-0.0007	0.9991	**0.9982**
5	0.0007	-0.0006	0.9990	**0.9980**

Carryover Test

Carryover test was assessed by injecting six replicates of matrix blank (COT BLANK), six replicates of extracted high concentrations of analyte(s) of the Calibration Curve range (COT ULOQ) and six extracted LLOQ concentrations of analyte(s) of the Calibration Curve range (COT LLOQ). Celecoxib carry over for six runs was found to be negligible (2.13, 0.00, 0.00, 0.00, 0.00 and 0.00 ng/mL). No significant carryover was observed at the RT of analyte as well as IS and the results (Table 2) are within the acceptable range of not greater than 20% of the LLOQ.

Table 2: Carry Over Test Results for Celecoxib in human plasma.

QC NO.	LLOQ		ULOQ					
	Analyte Area	IS Area	Analyte Area	IS Area	Analyte Area	% Carryover	IS Area	%Carryover
1	16085	2776475	8523232	3954188	401	2.13	0	0.00
2	15766	2625759	5253913	2433045	0	0.00	0	0.00
3	15133	2600711	5122034	2361810	0	0.00	0	0.00
4	20660	3479260	4810662	2215685	0	0.00	0	0.00
5	27247	4345893	5240070	2400740	0	0.00	0	0.00
6	18316	3119829	8843079	4112216	0	0.00	0	0.00
Mean	18867	3157987	6298831	2912947	0	0.00	0	0.00

Selectivity

a) Matrix selectivity

Eight human plasma lots were screened for selectivity and all eight lots including haemolytic and lipidemic plasma were found free of any significant interference for Celecoxib and internal standard.

b) Analyte selectivity

% Interference at RT of Celecoxib d7 was found to be 0.08. % Interference at RT of Celecoxib in presence of IS was found to be 0.09. %.

Sensitivity

Sensitivity was determined in terms of LLOQ, ‘Lower Limit of Quantification’. The lowest limit of reliable quantification for Celecoxib in human plasma was set at the concentration of the LLOQ 5.047 ng/mL. The precision and accuracy for Celecoxib at this concentration was found to be 2.55% and 105.38%.

Recovery

Extraction recovery of celecoxib from plasma was determined at 3 concentrations (HCQ, MQC2, and LQC). Six sets each of quality control samples was withdrawn from deep freezer and thawed or prepared freshly. These quality control samples (extracted samples) will be processed by adding the internal standard and injected along with the extracted samples. The internal standard response in extracted samples of LQC, MQC2 and HQC were compared with the response of internal standard in the entire non-extracted quality control sample. The mean recovery of celecoxib at HQC, LQC, and MQC2 was found to be 81.24% with a precision range of 0.49% to 1.16%. The mean recovery of internal standard Celecoxib d7 was 57.23% with a precision ranging from 1.77% to 3.91%. The results are represented in Table 3.

Table 3: Recovery of Celecoxib from Human Plasma.

	Celecoxib Response					
	Extracted QC	Spiked QC	Extracted QC	Spiked QC	Extracted QC	Spiked QC
S. No	LQC	LQC	MQC	MQC	HQC	HQC
1	33149	42523	2773710	3414633	4236097	5019658
2	33469	41651	2729553	3458440	4242185	4954049
3	33138	42340	2787092	3417134	4264054	5006801
4	33629	41755	2782319	3437046	4209110	5023718
5	32621	42667	2773828	3433550	4217519	5036593
6	33182	42848	2769938	3454836	4252492	5001566

Mean	**33198.0**	**42297.3**	**2769406.7**	**3435939.8**	**4236909.5**	**5007064.2**
S.D.	**345.94**	**490.82**	**20523.95**	**18318.83**	**20769.85**	**28805.60**
CV(%)	**1.04**	**1.16**	**0.74**	**0.53**	**0.49**	**0.58**
N	**6**	**6**	**6**	**6**	**6**	**6**
% Recovery	**78.49**		**80.60**		**84.62**	
Overall Recovery	**81.24 ± 3.115 S.D.**					

Matrix Effect

The matrix effect was determined for two concentrations LQC (15.143 ng/mL) and HQC (1917.869 ng/mL) by comparing peak areas of analyte-spiked plasma aliquots before extraction to peak areas of analyte-spiked solutions extracted from blank plasma and comparing the response of solutions spiked with analyte after extraction to the response of analyte dissolved in mobile phase. No significant matrix effect was observed in all the 11 replicates and the results are represented in Table 4.

Table 4: Matrix Effect of Celecoxib in human plasma in different lots (N=11).

	LQC				**HQC**			
Matrix Lot. No.	**Area of Analyte**	**Area of Internal Standard**	**Area Ratio**	**IS Normalized Matrix Factor**	**Area of Analyte**	**Area of Internal Standard**	**Area Ratio**	**IS Normalized Matrix Factor**
1	59912	5162051	0.0116	0.99	6717605	4879587	1.3767	0.99
2	59826	5218549	0.0115	0.98	6800926	4832036	1.4075	1.01
3	64856	5268090	0.0123	1.05	6798400	4896225	1.3885	1.00
4	60671	5220783	0.0116	0.99	6651663	4840300	1.3742	0.99
5	62389	5246322	0.0119	1.02	6757963	4856238	1.3916	1.00
6	61134	5250421	0.0116	0.99	6742333	4867325	1.3852	0.99
7	61853	5280664	0.0117	1.00	6768158	4843307	1.3974	1.00
8	60654	5192937	0.0117	1.00	6709749	4862458	1.3799	0.99
9	62066	5224944	0.0119	1.01	6828999	4921145	1.3877	1.00
N-lipomic	60930	5248125	0.0116	0.99	6796126	4903620	1.3859	0.99
O-Hemolised	60278	5227338	0.0115	0.98	6816020	4892288	1.3932	1.00
	SD			**0.0200**	**SD**			**0.0068**
	% CV			**2.00**	**% CV**			**0.68**
	N			**11**	**N**			**11**

Dilution integrity

The upper linearity concentration (ULOQ) can be extended upto 8579.941 ng/mL by ½ and ¼ dilutions with screened human blank plasma. The precision (%CV) and accuracy for two fold dilution was 1.55% and 90.31% respectively. Similarly, the precision and accuracy for four fold dilution was 1.03% and 90.88% respectively.

Ruggedness

One precision and accuracy batch (PA-2) was processed by the different analyst and analyzed using different column of same make and different set of solutions on the different instrument (BE-MS-05).Within batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC was 1.69%, 1.07%, 0.48%, 0.41%, and 0.47%, respectively. Within batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC was 106.93%, 104.93%, 106.97%, 91.90%, and 101.97%, respectively.

Re Injection Stability

In accessing the reinjection stability, six sets of QC samples (LQC and HQC) were processed and analyzed with calibration curve standard. The mean concentration of reinjected QCs was compared against the mean of the QCs when injected for first time. The results (Table 5) demonstrate that the reinjected samples were stable for 47 hours. The percent stability at 47 hours ranged from 100.32% to 101.49% and precision ranged 0.66% to 2.14%.

Table 5: Reinjection Stability Data for Celecoxib.

CELECOXIB	**Reinjection Stability (0 hour)**				**Reinjection Stability (47 Hours)**			
	LQC (ng/mL)		**HQC (ng/mL)**		**LQC (ng/mL)**		**HQC (ng/mL)**	
QC#	**15.143**	**% Accuracy**	**1917.869**	**% Accuracy**	**15.143**	**% Accuracy**	**1917.869**	**% Accuracy**
1	15.737	103.92	1964.869	102.45	16.307	107.68	1971.063	102.77
2	15.483	102.25	1957.979	102.09	15.758	104.06	1963.163	102.36
3	15.612	103.10	1957.941	102.09	15.505	102.39	1951.518	101.75
4	15.775	104.18	1892.526	98.68	16.280	107.51	1959.421	102.17

5	15.876	104.84	1952.058	101.78	15.698	103.67	1933.572	100.82
6	15.825	104.51	1967.161	102.57	16.165	106.75	1951.476	101.75
Mean	**15.7180**		**1948.7557**		**15.9522**		**1955.0355**	
S.D.	**0.14593**		**28.07318**		**0.34086**		**12.86774**	
C.V.%	**0.93**		**1.44**		**2.14**		**0.66**	
% Nominal	**103.80**		**101.61**		**105.34**		**101.94**	
N	**6**		**6**		**6**		**6**	
% Stability					**101.49**		**100.32**	

Stability studies

Stability of celecoxib and celecoxib d7 was investigated by injecting six replicates of prepared stock dilutions of celecoxib equivalent to final MQC2 quality control concentration and celecoxib d7 at final working concentration from spiking solution. Comparison of the mean area response of stability samples of Celecoxib and Celecoxib d7 at 16 hours 25 minutes was carried out against the comparison samples (freshly prepared). The precision of spiking solution stability of celecoxib at 0 hours and 16 hours 25 minutes was found to be 0.56% and 1.11% respectively. The percentage of stability was found to be 102.39%. The precision of room temperature (20 ± 5°C) spiking solution stability of celecoxib d7 at 0 hours and 16 hours 25 minutes was 0.32% and 1.56% respectively and percentage of stability was found to be 101.94%.

Stability in Whole Blood

12 mL each of whole blood was spiked with Celecoxib at a concentration of LQC (15.143 ng/mL) and HQC (1917.869 ng/mL). The six replicates of LQC and HQC level samples of plasma were taken and compared the response ratio of samples kept at room temperature for a period of minimum 3 hours 39 minutes with the fresh (Immediately centrifuged) samples. Results demonstrated that the whole blood samples were stable for 3 hours 39 minutes at room temperature (20±5 °C). The percent stability at LQC level was found to be 99.41% with the precision range of 1.31-2.05% and at HQC level was found to be 100.19% with the precision range of 0.49%-0.77% for 3 hours 39 minutes. The results are shown in Table 6.

Table 6: Stability of Celecoxib in Whole Blood for 3 hours 39 minutes.

S. NO.	LQC level		HQC level	
	Comparison samples	Stability sample	Comparison sample	Stability sample
	Area Ratio	Area Ratio	Area Ratio	Area Ratio
1	0.0113	0.0113	1.1996	1.2106
2	0.0116	0.0111	1.1960	1.1990
3	0.0112	0.0110	1.1987	1.2028
4	0.0111	0.0112	1.2073	1.2017
5	0.0110	0.0114	1.1921	1.2018
6	0.0115	0.0113	1.1910	1.1826
Mean	**0.01128**	**0.01122**	**1.19745**	**1.19975**
SD	**0.000232**	**0.000147**	**0.005925**	**0.009271**
% CV	**2.05**	**1.31**	**0.49**	**0.77**
N	**6**	**6**	**6**	**6**
%Stabilty	**99.41**		**100.19**	

Concomitant Drug effect

Six replicates of LLOQ QC, LQC and HQC samples were prepared in screened plasma spiked with concomitant drugs. Above quality control samples were processed and analyzed along with one bulk spiked calibration curve and quality control samples i.e. six replicates of LLOQ QC, LQC, and HQC samples. The results are shown in Table 7.

Table 7: Within Batch Precision and Accuracy of Celecoxib for Concomitant Drug Effect.

CELECOXIB	Nominal Concentration (ng/mL)					
CCDE QC#	LLOQ QC		LQC		HQC	
	5.149	% Accuracy	15.143	% Accuracy	1917.869	% Accuracy
1	5.441	105.68	15.994	105.62	1983.136	103.40
2	5.607	108.90	15.972	105.47	1965.826	102.50
3	5.472	106.28	15.760	104.08	1975.218	102.99
4	5.365	104.19	15.864	104.76	1970.694	102.75
5	5.360	104.11	15.642	103.30	1955.531	101.96

6	5.493	106.67	16.106	106.36	1971.190	102.78
Mean	**5.4563**		**15.8897**		**1970.2658**	
S.D.	**0.09181**		**0.16928**		**9.25235**	
C.V.%	**1.68**		**1.07**		**0.47**	
% Nominal	**105.97**		**104.93**		**102.73**	
N	**6**		**6**		**6**	

Precision and Accuracy

Accuracy and precision for the analyte were determined by analyzing QC's including the lower limit of quantification samples (HQC, MQC1, MQC2, LQC, LLOQ QC) with the standard calibration curve. Sets of samples were analysed within a single analytical run (within batch precision) and between different runs (between batch precision) on different days. Intra-batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC ranged from 1.33%-2.97%, 0.86%-1.77%, 0.29%-0.77%, 0.40%-1.06%, and 0.30%-1.44%, respectively. Intra-batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC ranged from 104.11%-110.72%, 103.80%-107.59%, 105.25%-108.89%, 91.90%-93.16%, and 101.61%-103.84%, respectively. Inter batch precision for LLOQ QC, LQC, MQC1, MQC2, and HQC was 2.28%, 1.10%, 0.79%, 0.66% and 0.59%, respectively. Inter batch accuracy for LLOQ QC, LQC, MQC1, MQC2, and HQC was 105.52%, 105.44%, 107.54%, 92.37%, and 102.41%, respectively.

RESULTS AND DISCUSSION

For quantification of celecoxib, sensitive MRM scan was employed using celecoxib d7 as IS in human plasma. Enhanced product ion spectra of celecoxib (Fig. 7A) and celecoxib d7 (Fig. 7B) show the protonated molecules of *m/z* 380 → 316 and 387.2 → 323.2 respectively. Calibration for the quantification of celecoxib was performed using the ratio of peak area of the analyte to that of IS. The ratio of peak area of the analyte to that of IS was proportional to analyte concentration from 5 - 2500 ng/mL for celecoxib. A linear regression model was used to describe the regression relationship, and 1/*x* was the best linear fit of the calibration curve (*r*2 = 0.99). The limit of detections (LOD: 1 ng/mL for celecoxib) were defined as the lowest concentration of analytes that are spiked into plasma and resulted in MRM signal that was 3 times greater than noise. Limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve that was measured with acceptable accuracy (within ± 20% of the theoretical value) and precision [coefficient of variation (CV) less than ± 20%] and the LOQ was 5 ng/mL for celecoxib. The precision of the assay was expressed as the CV%, which was calculated as percent of the standard deviation divided by the mean of observed concentrations. The results indicated that the method was accurate with excellent accuracy range of 91.9–110.72%, and the CV was within 10%. Matrix effect is a special phenomenon associated with LC–MS determination of drugs from biological fluids such as plasma and other matrices. Endogenous components extracted from plasma may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes. For this reason, the matrix effect was evaluated under the experimental conditions used in this study. As shown in Table 4, the average ion suppression or enhancement for celecoxib at low QC and high QC levels was < 3%, suggesting that matrix effect on the analysis was negligible. The stability results showed that celecoxib spiked into human plasma was stable for 18 h at ambient temperature, for 7 days at 4°C, and for 4 weeks at –20 and –70°C. Stability of the analyte in the sample is of crucial importance to the validity of the split sample program. Thus, proper storage of all samples is very important to obtaining reliable results and their interpretation in analysis.

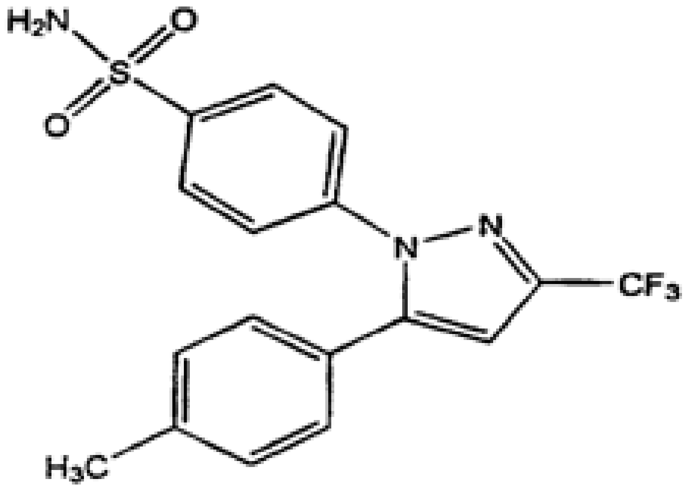
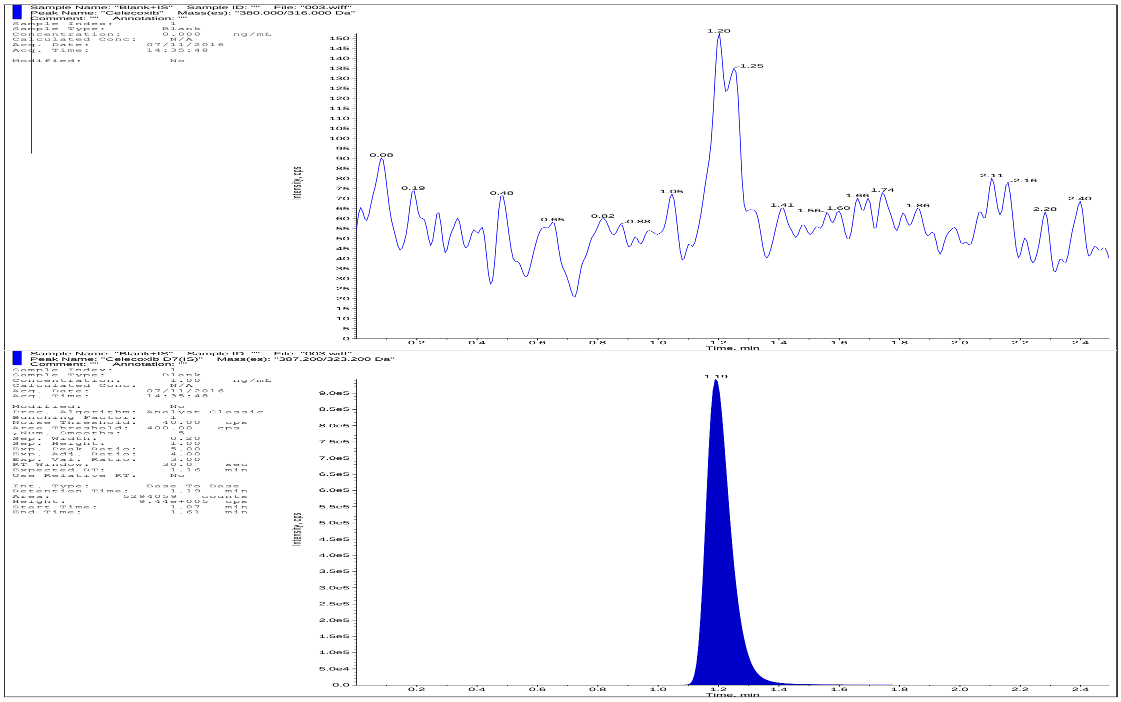


Figure 1: Structural formula of Celecoxib.



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Acq. Time:              14:35:48

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Exp. Val. Ratio:        3.00
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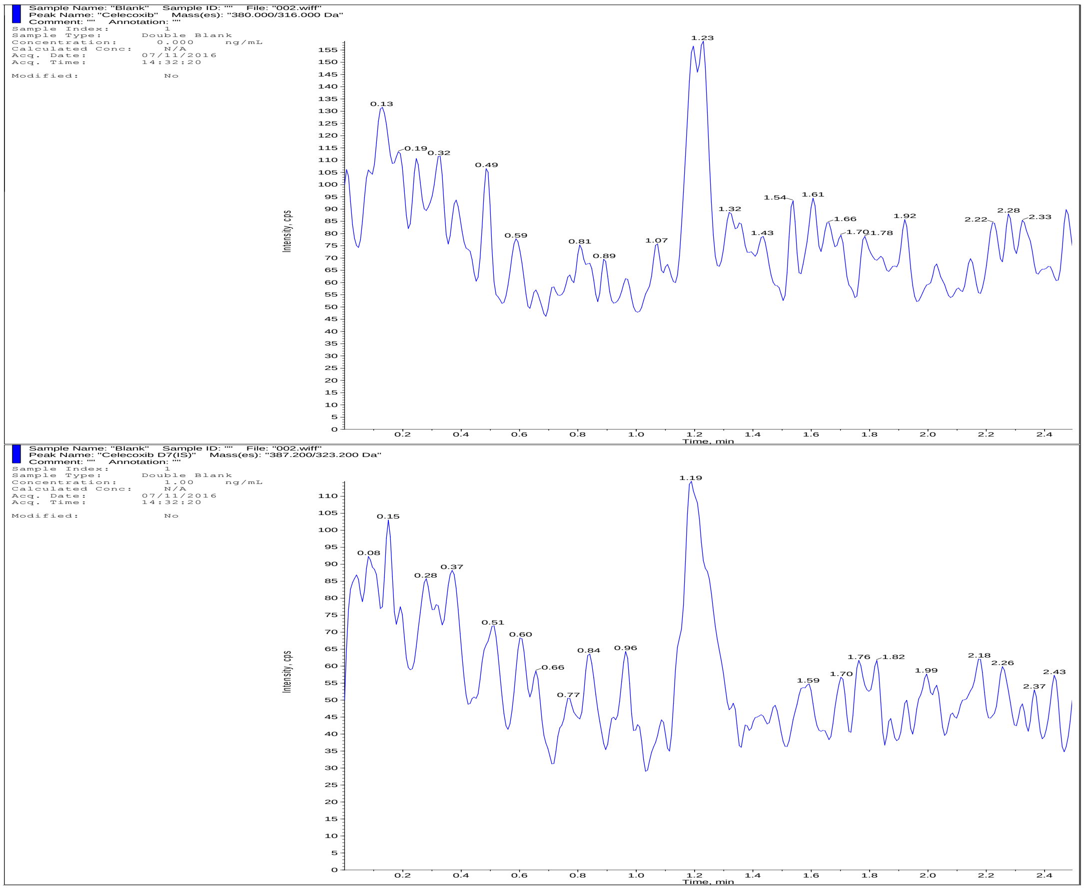
Figure 2: Typical MRM chromatograms of Blank human plasma.



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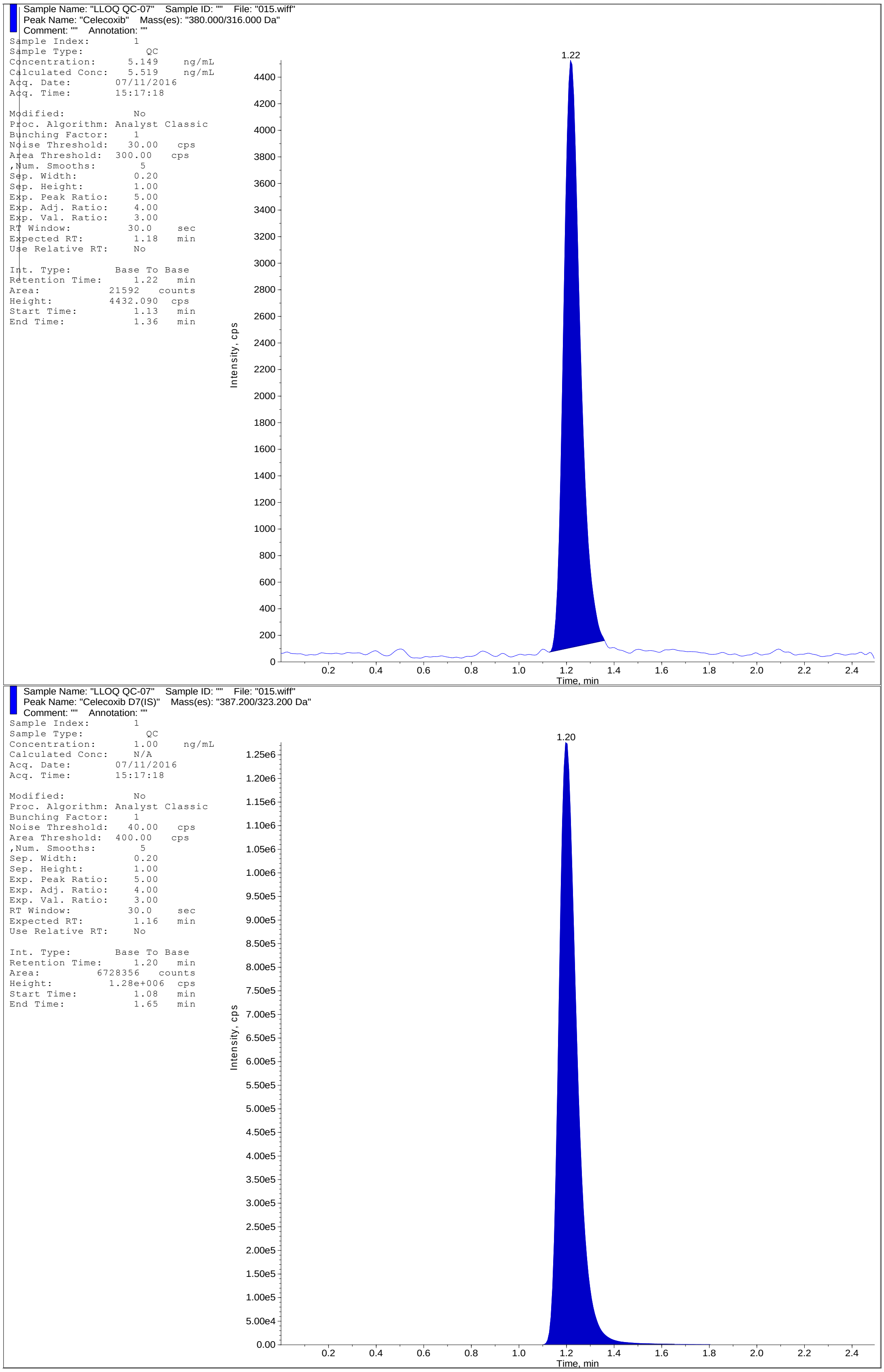
Peak Name: "Celecoxib"    Mass(es): "380.000/316.000 Da"
Comment: ""    Annotation: ""
Sample Index:           1
Sample Type:            Double Blank
Concentration:          0.000      ng/mL
Calculated Conc:        N/A
Acq. Date:              07/11/2016
Acq. Time:              14:32:20

Modified:               No
```

```
Sample Name: "Blank"    Sample ID: ""    File: "002.wiff"
Peak Name: "Celecoxib D7(IS)"    Mass(es): "387.200/323.200 Da"
Comment: ""    Annotation: ""
Sample Index:           1
Sample Type:            Double Blank
Concentration:          1.00       ng/mL
Calculated Conc:        N/A
Acq. Date:              07/11/2016
Acq. Time:              14:32:20

Modified:               No
```

Figure 3: Typical MRM chromatograms of Celecoxib & Celecoxib d7 (IS) in human plasma spiked at LLOQ level.



```
Sample Name: "LLOQ QC-07"    Sample ID: ""    File: "015.wiff"
Peak Name: "Celecoxib"    Mass(es): "380.000/316.000 Da"
Comment: ""    Annotation: ""
Sample Index:         1
Sample Type:             QC
Concentration:        5.149    ng/mL
Calculated Conc:      5.519    ng/mL
Acq. Date:         07/11/2016
Acq. Time:         15:17:18

Modified:             No
Proc. Algorithm: Analyst Classic
Bunching Factor:      1
Noise Threshold:    30.00    cps
Area Threshold:   300.00    cps
,Num. Smooths:         5
Sep. Width:           0.20
Sep. Height:          1.00
Exp. Peak Ratio:      5.00
Exp. Adj. Ratio:      4.00
Exp. Val. Ratio:      3.00
RT Window:           30.0     sec
Expected RT:          1.18    min
Use Relative RT:      No

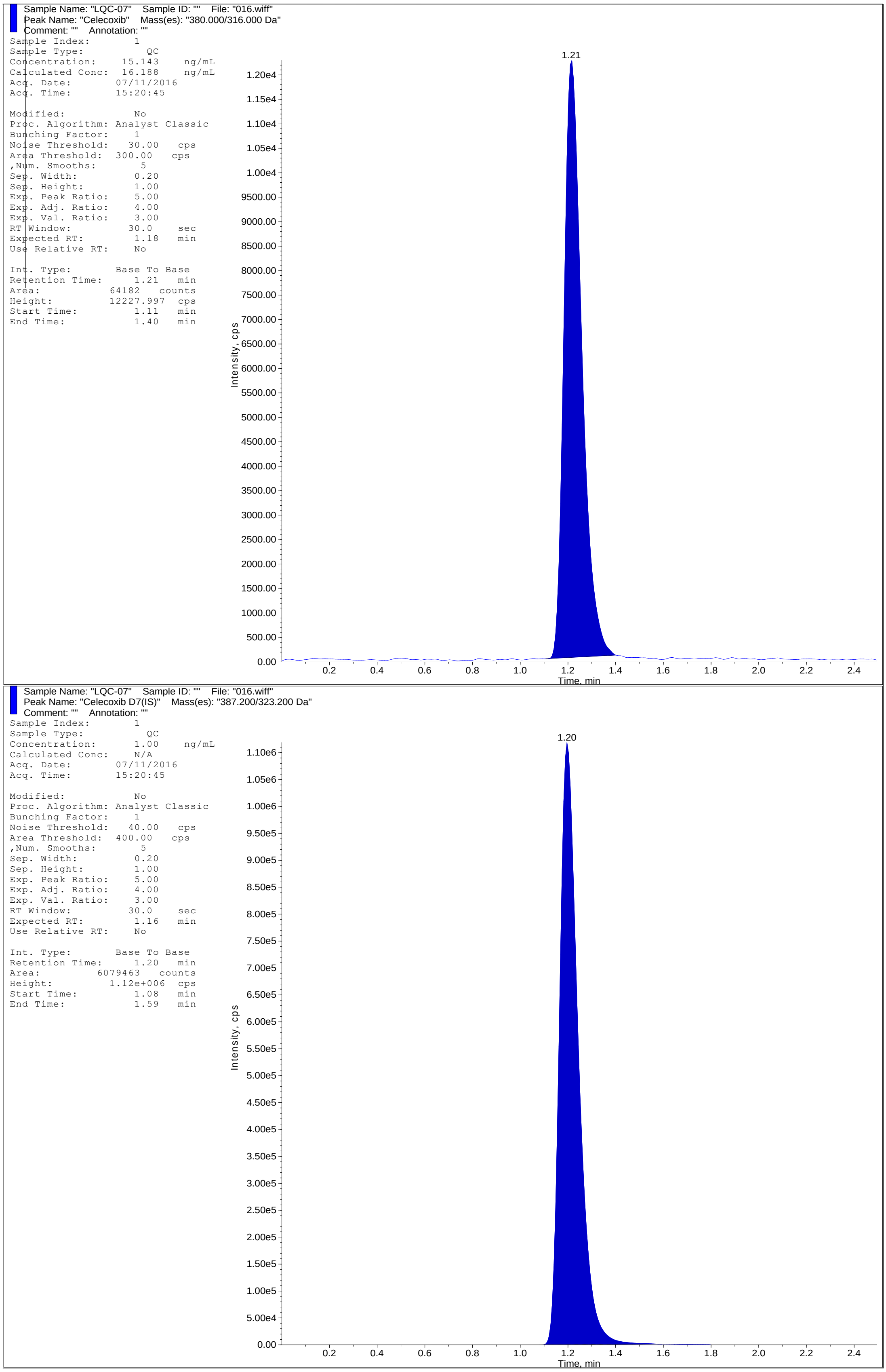
Int. Type:         Base To Base
Retention Time:       1.22    min
Area:              21592    counts
Height:            4432.090   cps
Start Time:           1.13    min
End Time:             1.36    min
```

```
Sample Name: "LLOQ QC-07"    Sample ID: ""    File: "015.wiff"
Peak Name: "Celecoxib D7(IS)"    Mass(es): "387.200/323.200 Da"
Comment: ""    Annotation: ""
Sample Index:         1
Sample Type:             QC
Concentration:        1.00     ng/mL
Calculated Conc:      N/A
Acq. Date:         07/11/2016
Acq. Time:         15:17:18

Modified:             No
Proc. Algorithm: Analyst Classic
Bunching Factor:      1
Noise Threshold:    40.00    cps
Area Threshold:   400.00    cps
,Num. Smooths:         5
Sep. Width:           0.20
Sep. Height:          1.00
Exp. Peak Ratio:      5.00
Exp. Adj. Ratio:      4.00
Exp. Val. Ratio:      3.00
RT Window:           30.0     sec
Expected RT:          1.16    min
Use Relative RT:      No

Int. Type:         Base To Base
Retention Time:       1.20    min
Area:            6728356    counts
Height:          1.28e+006   cps
Start Time:           1.08    min
End Time:             1.65    min
```

Figure 4: Typical MRM chromatograms of Celecoxib & Celecoxib d7 (IS) in human plasma spiked at LQC level.



```
Sample Name: "LQC-07"    Sample ID: ""    File: "016.wiff"
Peak Name: "Celecoxib"    Mass(es): "380.000/316.000 Da"
Comment: ""    Annotation: ""
Sample Index:        1
Sample Type:           QC
Concentration:    15.143    ng/mL
Calculated Conc:  16.188    ng/mL
Acq. Date:        07/11/2016
Acq. Time:        15:20:45

Modified:            No
Proc. Algorithm: Analyst Classic
Bunching Factor:     1
Noise Threshold:   30.00   cps
Area Threshold:  300.00   cps
,Num. Smooths:        5
Sep. Width:          0.20
Sep. Height:         1.00
Exp. Peak Ratio:     5.00
Exp. Adj. Ratio:     4.00
Exp. Val. Ratio:     3.00
RT Window:          30.0     sec
Expected RT:         1.18    min
Use Relative RT:     No

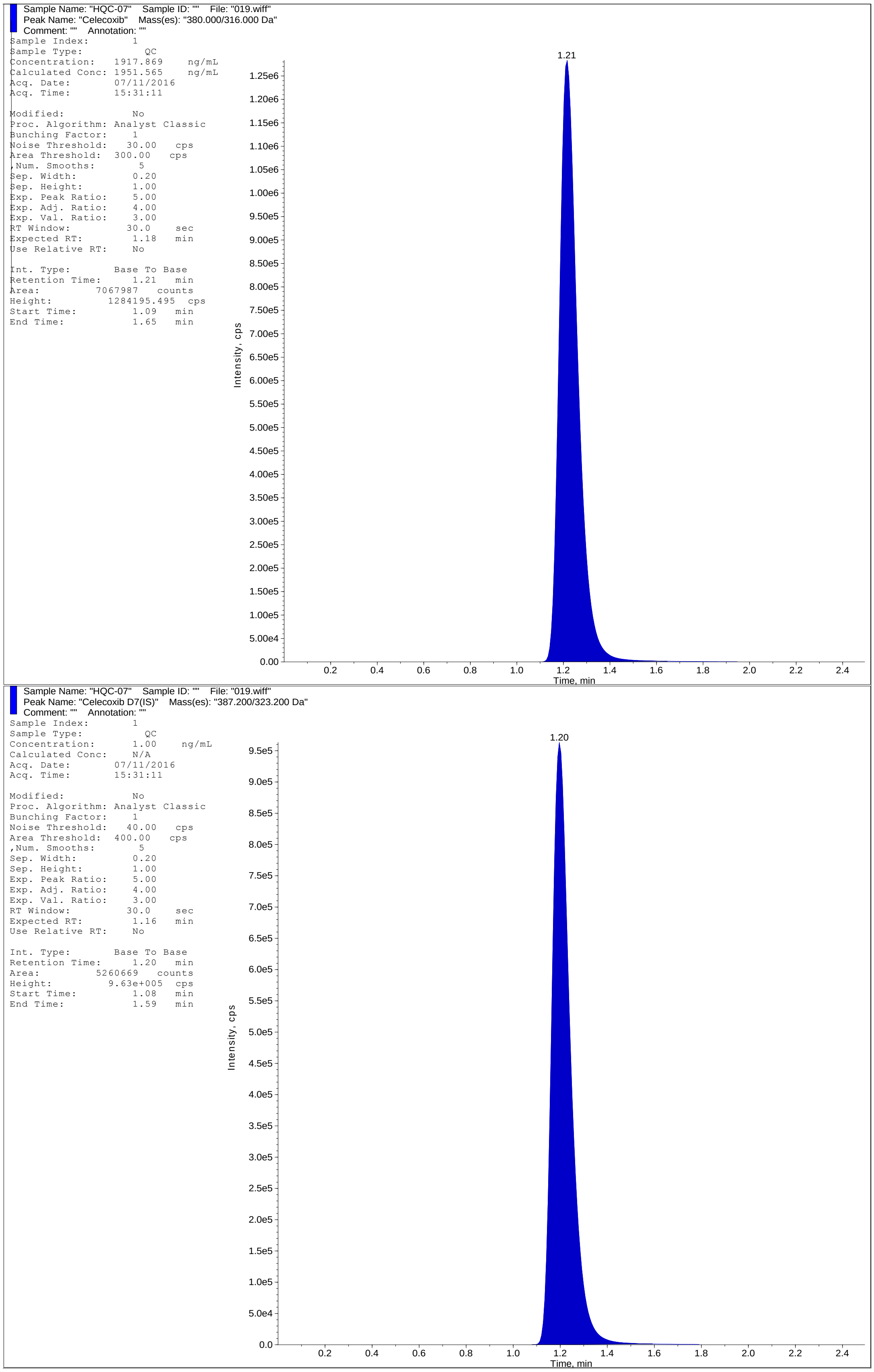
Int. Type:        Base To Base
Retention Time:      1.21    min
Area:            64182    counts
Height:          12227.997   cps
Start Time:          1.11    min
End Time:            1.40    min
```

```
Sample Name: "LQC-07"    Sample ID: ""    File: "016.wiff"
Peak Name: "Celecoxib D7(IS)"    Mass(es): "387.200/323.200 Da"
Comment: ""    Annotation: ""
Sample Index:        1
Sample Type:           QC
Concentration:       1.00    ng/mL
Calculated Conc:     N/A
Acq. Date:        07/11/2016
Acq. Time:        15:20:45

Modified:            No
Proc. Algorithm: Analyst Classic
Bunching Factor:     1
Noise Threshold:   40.00   cps
Area Threshold:  400.00   cps
,Num. Smooths:        5
Sep. Width:          0.20
Sep. Height:         1.00
Exp. Peak Ratio:     5.00
Exp. Adj. Ratio:     4.00
Exp. Val. Ratio:     3.00
RT Window:          30.0     sec
Expected RT:         1.16    min
Use Relative RT:     No

Int. Type:        Base To Base
Retention Time:      1.20    min
Area:          6079463    counts
Height:          1.12e+006   cps
Start Time:          1.08    min
End Time:            1.59    min
```

Figure 5: Typical MRM chromatograms of Celecoxib & Celecoxib d7 (IS) in human plasma spiked at MQC level.



```
Sample Name: "HQC-07"    Sample ID: ""    File: "019.wiff"
Peak Name: "Celecoxib"    Mass(es): "380.000/316.000 Da"
Comment: ""    Annotation: ""
Sample Index:        1
Sample Type:           QC
Concentration:    1917.869    ng/mL
Calculated Conc:  1951.565    ng/mL
Acq. Date:        07/11/2016
Acq. Time:        15:31:11

Modified:            No
Proc. Algorithm:  Analyst Classic
Bunching Factor:     1
Noise Threshold:    30.00    cps
Area Threshold:   300.00    cps
,Num. Smooths:         5
Sep. Width:          0.20
Sep. Height:         1.00
Exp. Peak Ratio:     5.00
Exp. Adj. Ratio:     4.00
Exp. Val. Ratio:     3.00
RT Window:          30.0     sec
Expected RT:         1.18    min
Use Relative RT:     No

Int. Type:        Base To Base
Retention Time:      1.21    min
Area:          7067987    counts
Height:         1284195.495  cps
Start Time:          1.09    min
End Time:            1.65    min
```

```
Sample Name: "HQC-07"    Sample ID: ""    File: "019.wiff"
Peak Name: "Celecoxib D7(IS)"    Mass(es): "387.200/323.200 Da"
Comment: ""    Annotation: ""
Sample Index:        1
Sample Type:           QC
Concentration:       1.00    ng/mL
Calculated Conc:     N/A
Acq. Date:        07/11/2016
Acq. Time:        15:31:11

Modified:            No
Proc. Algorithm:  Analyst Classic
Bunching Factor:     1
Noise Threshold:    40.00    cps
Area Threshold:   400.00    cps
,Num. Smooths:         5
Sep. Width:          0.20
Sep. Height:         1.00
Exp. Peak Ratio:     5.00
Exp. Adj. Ratio:     4.00
Exp. Val. Ratio:     3.00
RT Window:          30.0     sec
Expected RT:         1.16    min
Use Relative RT:     No

Int. Type:        Base To Base
Retention Time:      1.20    min
Area:          5260669    counts
Height:          9.63e+005  cps
Start Time:          1.08    min
End Time:            1.59    min
```

Figure 6: Typical MRM chromatograms of Celecoxib & Celecoxib d7 (IS) in human plasma spiked at HQC level.

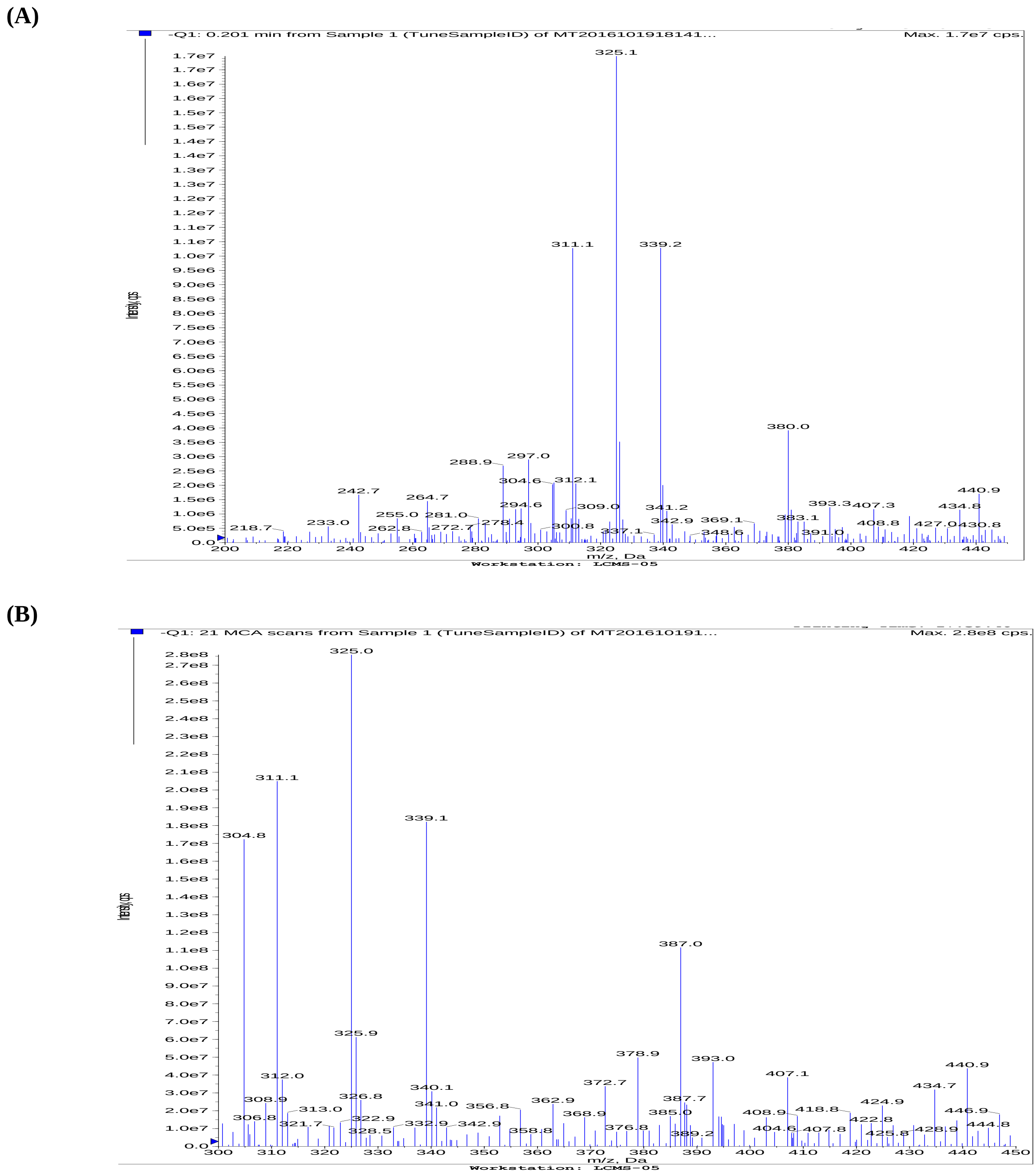


Figure 7: Product ion spectra of Celecoxib (A) and Celecoxib d7 (B).

Estimation of measurement uncertainty

Measurement uncertainty (MU) is a parameter that describes the range of values which the measurand can be declared to lie within a specified level of confidence because any measurement does not have a fixed known value. Measurement uncertainty can be estimated using laboratory quality control samples (LQCS) as previously demonstrated. In this study, MU was estimated using 25 QCS. As shown in Table 8, standard uncertainty value was 5.6 with 24 degrees of freedom. The MU estimation for celecoxib at 95% confidence interval with a coverage factor of *k* = 2 was 11.2%. Thus, an estimated quantitative value of 400 ng/mL, celecoxib, as an example, would be reported as 400 ± 400 × 11.2% ng/mL (400 ± 44.8 ng/mL). Thus, the true value of celecoxib in the example analyzed lies between 355.2 and 444.8 ng/mL. Measurement uncertainty should only be reported at the request of the client in cases involving a commercial or contract laboratory.

Table 8: Measurement Uncertainty Results of Celecoxib.

Parameter	**Value units (%)**	**Advisor**	**Standard Uncertainty**	**Degrees of Freedom (n-1)**
U_1 (Combined Uncertainty)	5.6%	$\sqrt{U_1^2} = 5.6$	5.6	24
Expanded Uncertainty(k= 2.0) n= 25		5.6 x 2.0 = 11.2		

CONCLUSIONS

A highly selective and sensitive LC–MS method for screening, confirmation and quantification of celecoxib in human plasma was developed and validated. For quantification, good linearity, accuracy, and precision were achieved. Stabilities of celecoxib at various storage conditions were determined. Measurement uncertainty was also estimated for this method. The method is capable of providing defensible evidence for identification of celecoxib and celecoxib d7 in human plasma. This innovative method offers several advantages for assaying Celecoxib in human plasma including simplicity, cost effectiveness, with high sensitivity and selectivity and reliably reproducible. The results from this validation study illustrated that this method can be used for determination of further pharmacological and toxicological profiles of celecoxib.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

All authors declare no conflicts of financial, personal or institutional interest in the preparation of this scientific article.

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