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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF IN COLCHICINE HUMAN PLASMA BY USING UPLC-MS/MS

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ABSTRACT

A sensitive and specific ultra-performance liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (UPLC-MS/MS) method, operating in the positive ionization mode, for the determination of Colchicine in human plasma using Colchicine D6 as internal standard (IS) was developed and validated. The analyte and IS were extracted by simple liquid-liquid extraction with a mixture of nHexane: Dichloromethane: Isopropyl Alcohol (60:30:10, v/v/v). The chromatographic separation was performed on a Purospher RP 18 (100×4.6 mm, 5µm) analytical column under isocratic conditions using a mixture of Acetonitrile: 0.05% Ammonia (90:10, v/v) as mobile phase at a flow rate of 0.500 mL/min. Total chromatographic run time was 3.0 min. Detection was performed on a XEVO TQ-S mass spectrometer by Waters. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent product ion transitions of m/z 400.1→358.2 for Colchicine and m/z 406.1→362.2 for IS respectively. The method was validated over the concentration range 0.04-7.56 ng/mL, with a coefficient of determination (r^2) of 0.9968. Limit of detection and limit of quantification were found 40.85 pg and 113.01 pg respectively. The retention time for Colchicine and IS were 2.12 min and 2.11 min respectively and overall chromatography run time was 3.0 minutes. The mean recovery of Colchicine and internal standard is 57.11% and 55.39% respectively. The method was validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. The intra- and inter-day precision and accuracy values were found to be within the assay variability limits as per the FDA guidelines.

Keywords *– Colchicine, Colchicine D6, UPLC-MS/MS, Human plasma, Linearity.*

1. INTRODUCTION

Colchicine is recommended for the treatment of acute gout flares in adults and also for prophylaxis of gout flares in young patients. It is a tricyclic alkaloid found in flowering plants *Colchicum autumnale* (meadow saffron) and *Gloriosa superb* (glory lily) and has anti-

inflammatory properties. The chemical name of Colchicine is N-[(7*S*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide. Its molecular formula is $C_{22}H_{25}NO_6$ and molecular weight 399.44 g/mol. The molecular structure of Colchicine and Colchicine D6 were shown in figure 1 and 2 respectively.

Colchicine is freely soluble in Chloroform and Methanol with mean pKa value of 5.9. It is metabolized by cytochrome P450 (CYP) 3A4 in vitrointo two primary metabolites, 2-*O*-demethyl colchicine and 3-*O*-demethyl colchicine. The total plasma concentration of these metabolites is less than 5% of that of the parent drug.

Literature survey revealed that numerous HPLC and LC-MS/MS methods have been reported for estimation of Colchicine in human plasma. Among chromatographic techniques; the LC-MS/MS was widely used for the analysis. In the present work a highly sensitive, selective and high throughput UPLC-MS/MS method has been developed and validated as per USFDA guidelines. The method offers higher sensitivity, and small turnaround time for analysis using 300µL human plasma for liquid-liquid extraction. Picogram quantities of Colchicine were determined from human plasma with acceptable accuracy and precision. Precise and quantitative recovery with minimal matrix effect was obtained at all quality control levels. The method is readily applicable to human pharmacokinetic studies.

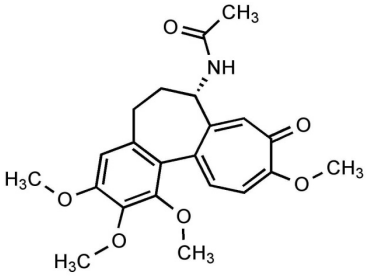

Figure 1: Molecular structure of Colchicine

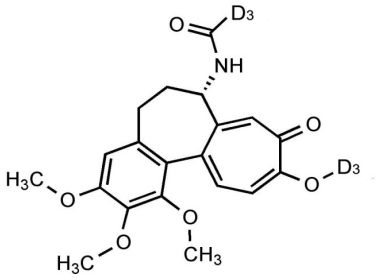

Figure 2: Molecular structure of Colchicine D6 (internal standard)

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Colchicine (batch no: CS-CH-345) and Colchicine D6(batch no: CS-CC-447) were produced from Clearsynth Labs, Ltd. (Mumbai, India). Acetonitrile (HPLC grade), Isopropyl Alcohol and Liquor Ammonia were obtained from Fisher Scientific (Mumbai, India). nHexane, Dichloromethane and Ortho Phosphoric acid were supplied by Merck Scientific Pvt, Ltd. (Mumbai, India). Methanol (HPLC grade) was from Ultra International (Bangalore, India). Water used in the entire analysis was prepared using Milli-Q water purification system from Millipore.The human plasma was obtained from Micro Therapeutic Research Labs Private Ltd., Chennai, India.

2.2 Preparation of Solutions

The buffer solution consisting of 50% Ortho Phosphoric acid in Milli-Q water was prepared. The extraction solvent consisting of nHexane: Dichloromethane: Isopropyl Alcohol (60:30:10, v/v/v) and mobile phase of Acetonitrile: 0.05% Ammonia (90:10, v/v) were prepared and filtered through nylon (0.22µm) membrane sample filter paper and degas. All solutions were stored at ambient temperature.

2.3 Preparation of Drug Stock Solution

The standard stock solution of Colchicine and IS (1mg/mL) was prepared by dissolving 2 mg of drug/IS, transferring to 2.0 mL of volumetric flask containing 1.0 mL of Methanol and make up the final volume with the same. While the working solution (pg/mL) was prepared in acetonitrile: water (80:20, v/v) and stored in refrigerator between 2-8 °C.

2.4 Preparation of Standard Blank and Zero Standard Samples

The standard blank samples were prepared by transferring 300 µL screened blank plasma into pre-labeled vials. The standard zero was prepared by adding 50 µL of internal standard solution (25 ng/mL) into 300 µL screened blank plasma into a pre-labeled vial and vortex for few seconds.

2.5 Human Plasma Extraction

Samples were extracted in the following manner**:** To 300 µL of human plasma mixed with K_2EDTA anticoagulant, 50 µL IS (25 ng/mL) was added and vortex well. Then 250 µL of buffer (50% Ortho Phosphoric acid in water) was added and vortexed for few minutes. 2.5 mL of extraction solvent (nHexane: Dichloromethane: Iso Propyl Alcohol, 60:30:10, v/v/v) was added and samples were kept for vibramax at 2000 rpm for 10 minutes. Then the samples were centrifuged at 3500 rpm at 4°C for 5 min. The supernatant was transferred into polypropylene tubes and evaporated to dryness at 40°C at 15 psi for 18 minutes under nitrogen evaporator.Finally, samples were reconstituted with 200 µL of mobile phase (Acetonitrile: 0.05% Ammonia in water, 90:10, v/v) and vortex for few minutes. Samples were then transferred into auto-injector vials and analysed in UPLC- MS/MS.

2.6 Liquid Chromatographic and Mass Spectrometric Conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of Colchicine and IS was performed on a Purospher RP 18 (100×4.6 mm, 5 µm) column and maintained at 40°C in a column oven. The mobile phase consisted of Acetonitrile: 0.05% Ammonia (90:10, v/v). The flow rate of the mobile phase was kept at 0.500 mL/min. Ionization and detection of Colchicine and IS was carried out on a Waters Quattro Premier XE (USA) triple quadrupole mass spectrometer, equipped with

Electro spray ionization and operating in positive ionization mode. The source dependent and compound dependent parameters optimized for Colchicine and IS are shown in Table 1.

Table 1: MS/MS source dependant and compound dependant parameters for Colchicine and Colchicine D6

Parameters		Set value
Cone gas flow (L/h)		150
Desolvation gas flow (L/h)		1000
Capillary voltage (kV)		3.10
Source temperature (°C)		150
Desolvation temperature (°C)		500
Extractor voltage (V)		5
Pressure of collision gas (psi)		6500
Cone voltage (V)	Colchicine	25
	Colchicine D6	25
Collision energy (eV)	Colchicine	20
	Colchicine D6	20
Dwell time (s)		0.2
Mode of analysis		Positive
MRM ion transition (m/z)	Colchicine	400.1/358.2
	Colchicine D6	406.1/362.2

2.7 Data Processing

The chromatograms were acquired and the data was processed by peak area ratio method using MassLynx 4.1 software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked calibration standard with the reciprocal of the drug concentration ratio as a weighting factor ($1/X^2$).

y = mx + b

Where,

y = peak area ratio of Colchicine to internal standard

m = slope of the calibration curve

x = concentration of Colchicine

b = y-axis intercept of the calibration curve

3. RESULTS

The validation was performed to evaluate the method in terms of the parameters e.g., linearity response, sensitivity, selectivity, precision and accuracy (within-batch and between-batch/inter-day), stabilities (freeze-thaw, bench top, short-term and long-term stock solutionsand long term stability in matrix), carryover effects, recovery, dilution integrity, matrix effect, matrix factor, autosampler stability and ruggedness experiment.

3.1 System Suitability

System suitability experiment was performed by injecting six consecutive injections using aqueous MQC solutions at the beginning of analytical batch or before any re-injection and signal to noise ratio meet the acceptance criteria.

3.2 Carryover effect

The carryover effect of the autosampler was performed by injecting sequence of injections during the start of the method validation and there was no significant injector carryover observed.

3.3 Separation, Specificity/Selectivity and Sensitivity

Selected blank human plasma from six different sources and were carried through the extraction procedure and chromatographed to determine the extent to which endogenous human plasma components may contribute to chromatographic interference with the Colchicine or internal standard. One hemolyzed, one heparinized and one lipidemic plasma were also checked for specificity along with this run. The retention time of Colchicine and internal standard were approximately 2.12 and 2.11 minutes, respectively. No significant interference was observed in six different lots of human plasma, hemolyzed plasma, heparinized plasma and lipidemic plasma samples. The peaks were completely separated and there was no interference peaks from endogenous substances. The Colchicine lower limit of quantitation was 0.04 ng/mL.

3.4 Matrix Factor

Samples of the relevant biological matrix from nine different sources which include six normal lot, one hemolyzed lot, lipidemic lot and one heparinized lot) were collected. The lower and higher calibration standard samples from each source were prepared and injected along with the six replicates of aqueous lower and higher calibration standard level concentrations. The %CV of matrix factor for Colchicine and internal standard were 11.86 and 12.52 respectively.

3.5 Calibration of Standard Curve (Linearity and Range)

The calibration curve was constructed between peak height ratios of Colchicine to the internal standard against Colchicine concentrations as given in Figure 4. Linearity was demonstrated by multiple analysis of spiked plasma sample containing Colchicine between 0.04 to 7.56 ng/ml calibration ranges. The regression coefficient (r^2) is 0.9968. A good linear relationship with the coefficient

of determination (r^2) of more than 0.99 was employed for determining of Colchicine concentration in plasma. Back calculations were made from the calibration curves to determine Colchicine of each calibration standard. The lower limit of quantification (LLOQ) was established at 0.04 ng/mL with the coefficient of variation of 9.61% indicates the sensitivity of the method.

3.6 Precision and Accuracy

3.6.1 Between–run accuracy and precision

The between-run accuracy and precision evaluation were assessed by the repeated analysis of human plasma samples containing different concentrations of Colchicine on separate occasions. A single run consisted of a calibration curve, six replicates of lower limit of quantitation (LLOQ), low, intermediate, medium and high quality control samples and results are expressed as the percentage of accuracy of the analytical method presented in Table 2. The between-run % coefficient of variation ranged from 1.31 to 12.22. The between-run % of accuracy value was ranged from 94.28 to 103.88 respectively.

3.6.2 Within-run accuracy and precision

Analyzing replicate concentrations of Colchicine in human plasma performed within-run accuracy and precision evaluations. The run consisted of a calibration curve plus a total of 30 spiked samples, including six replicates each of the lower limit of quantitation (LLOQ), low, intermediate, medium and high quality control samples. The between-run (inter-day) precision and accuracy of the assay procedure are shown in Table 3. The within-run % coefficient of variation ranged from 1.00 to 4.19. The within-run % of accuracy value was ranged from 96.91 to 114.10 respectively.

Table 2: Between-run precision and accuracy of the analytical method for Colchicine in plasma

Nominal concentration of Colchicine (pg/mL)	Mean concentration of Colchicine (pg/mL)	% CV	% Accuracy
HQC (5650.33)	5845.13	2.80	103.45
MQC (2925.17)	2833.67	1.31	102.07
INTQC (565.03)	541.00	4.55	95.75
LQC (113.01)	106.54	2.25	94.24
LLOQ QC (40.85)	42.44	12.22	103.88

Table 3: Within-run precision and accuracy of the analytical method for Colchicine in plasma

Nominal concentration of Colchicine (pg/mL)	Mean concentration of Colchicine (pg/mL)	% CV	% Accuracy
HQC (5650.33)	6416.79	1.76	113.56
MQC (2925.17)	3223.64	2.63	114.10
INTQC (565.03)	547.58	1.00	96.91
LQC (113.01)	118.70	3.96	105.04
LLOQ QC (40.85)	40.05	4.19	98.05

3.7 Recovery

The % recovery was calculated from ratio of area of extracted to un-extracted samples at each level are shown in Table 4. The six un-extracted samples each of low, medium and high quality control samples were prepared. The recovery results were based on a comparison of the UPLC -MS/MS response from plasma (un-extracted) to those from extracted plasma blank. The mean % recovery of Colchicine for LQC, MQC and HQC are 66.32, 51.67 and 53.35 respectively.

The % mean internal standard recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at LQC, MQC and HQC concentration against the mean peak area of internal standard in the un-extracted quality control samples at LQC, MQC and HQC concentrations. The mean % recovery for internal standard is 55.39.

Table 4: Recovery of Colchicine in plasma

Nominal concentration of Colchicine (pg/mL)	Extracted Colchicine response	Un-Extracted Colchicine response	% Recovery
HQC (5650.33)	78278	146737	53.35
MQC (2925.17)	38784	75065	51.67
LQC (113.01)	1573	2372	66.32

3.8 Dilution Integrity

Dilution quality control was diluted second and fourth times in human plasma. Prior to extraction, six samples each of second and fourth diluted samples were processed and analyzed with freshly processed calibration samples. The calculated concentrations, including the dilution factor for $1/2^{nd}$ and $1/4^{th}$ yielded coefficient of variation of 0.53 and 1.73 respectively. Percentage of nominal values for dilution factor $1/2^{nd}$ and $1/4^{th}$were 102.67 and 101.87 respectively.

3.9 Ruggedness

Different analyst with different column defines ruggedness. The run consisted of a calibration curve and a total of 30 spiked samples, including 6 replicate each of the lower limit of quantitation (LLOQ), low, intermediate, medium and high quality control samples. The % coefficient of variation ranged from 1.86 to 9.85 and the percentage of nominal values ranged from 95.92 to 107.04.

3.10 Stock Solution Stability

3.10.1 Short term stock solution stability at room temperature

Stock solution each of Colchicine and internal standard were stable after approximately 24.5 hours and at room temperature. For Colchicine and internal standard, the percentage stability was 94.90 and 93.89 respectively.

3.10.2 Long term stock solution stability in refrigerator between 2-8°C

Solution each of Colchicine at working curve standard level and internal standard solution at working internal standard level were stable for 90 days. For Colchicine and internal standard, the percentage stability was 93.99 and 95.67 respectively.

3.11 Bench Top Stability

The bench top stability samples each of low and high QC (stability samples) was kept on bench at room temperature was found stable at approximately 18.00 hours (Figure 5). The % change for LQC and HQC were -2.01 and -0.44 respectively.

Table 5: Bench top stability in plasma at room temperature

Bench top stability QCs			
Nominal concentration of Colchicine (pg/mL)	Mean concentration of Colchicine (pg/mL)	% CV	% Nominal
HQC (5650.33)	5858.91	1.98	103.69
LQC (113.01)	115.26	2.69	102.00
Comparison QCs			
HQC (5650.33)	5883.75	2.02	104.13
LQC (113.01)	117.54	2.76	104.01

3.12 Freeze and Thaw Stability (at -20 ±5 ºC)

The freeze and thaw stability samples each of LQC and HQC were found to be stable in human plasma after four freeze and thaw cycles (at -20 ±5°C). The % change for LQC and HQC were -2.15 and -1.51 respectively.

3.13 Autosampler Stability

Autosampler stability was evaluated by re-injecting accepted precision & accuracy batch, which were stored preferably in autosampler for 33.00 hours. The stability samples each of LQC and HQC was found to be stable in autosampler (at 5±3ºC). The % change for LQC and HQC were -1.76 and -0.66 respectively.

3.14 Long Term Stability of Colchicine and Internal Standard in Human Plasma

Process and analyze freshly spiked calibration curve standards and six replicates of each LQC and HQC samples and internal standard solutions for first day stability assessment and stability was assessed by comparing the stability samples against first day samples. The % mean long term working solution stability for Colchicine and internal standard samples were in the range of 90-110% and % CV was within 15%. The result suggest that Colchicine was stable in human plasma at -20 ±5°C and -70 ±5°Cupto 4 months given in Table 6.

Table 6: Long term stability of Colchicine and internal standard in human plasma

Store at -20ºC			
Nominal concentration of Colchicine (pg/mL)	Mean concentration of Colchicine (pg/mL)	% CV	% Nominal
HQC (5650.33)	5637.53	2.63	99.77
LQC (113.01)	110.60	5.98	97.87
Store at -70°C			
HQC (5650.33)	5723.15	3.18	101.29
LQC (113.01)	114.16	4.23	101.02

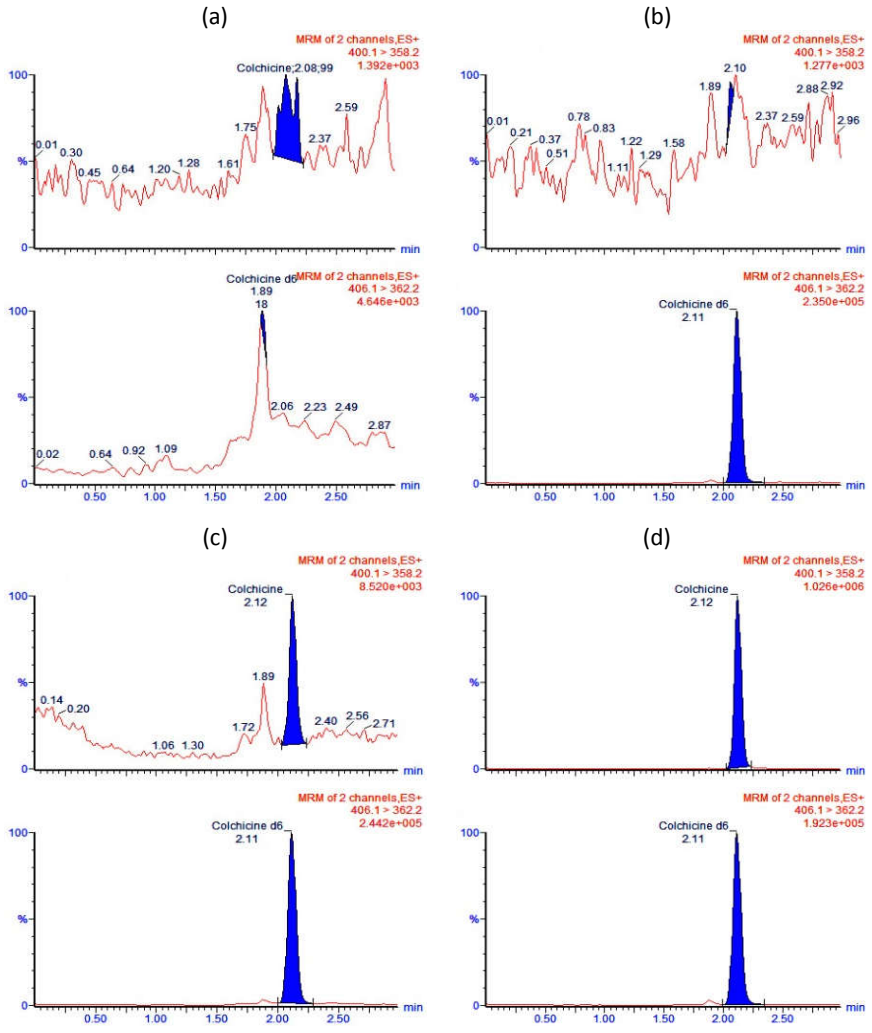

Figure 3:MRM ion-chromatograms of (a) blank plasma (without IS) (b) blank plasma with IS (c) Colchicine at LLOQ and IS (d) Colchicine at ULOQ and IS.

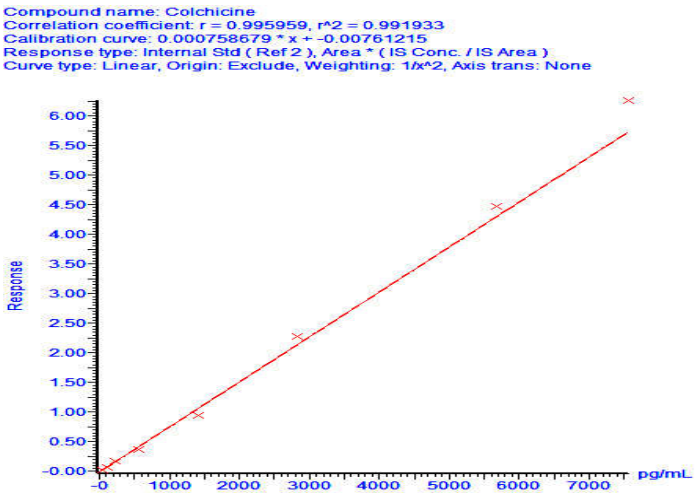

Figure 4: Calibration curve of Colchicine in human plasma analyzed by UPLC -MS/MS.

4. DISCUSSION

A rapid, sensitive and rugged liquid-liquid extraction ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was developed for determination of Colchicine in human plasma. The analysis was performed on a triple-quadrupole tandem mass spectrometer by multiple reactions monitoring mode via electrospray ionization. Different mobile phases were tried but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase Acetonitrile and 0.05% Ammonia (90:10%, v/v). The retention time of Colchicine and Colchicine D6(IS) were found to be 2.12 and 2.11 minutes and overall run times 3.0 minutes indicates a good baseline (Figure 3). The calibration curve for Colchicine obtained by plotting peak area ratio versus concentrations was found to be constituency accurate and precise over the 0.04-7.56 ng/mL calibration ranges with regression coefficient (r^2) ≥0.99. The coefficient of variation of response ratio of Colchicine and Colchicine D6 (IS) stock solutions were <5% respectively and no significant injector carry-over effects were observed. No significance interference was observed in system performance experiment determined the good selectivity and specificity of the Colchicine and internal standard during the extraction process and plasma matrix. The %CV of matrix factor of Colchicine and internal standard was less than 15% shows the absence of additional peaks indicates no interference of the endogenous substances in plasma matrix. The high percentage of recovery of Colchicine was found to be 66.32% indicates that the proposed method is accurate. The percentage accuracy and precision studies obtained were less than 15% for QC sample and less than 20% for LOQQC samples revealed that developed method was accurate and precise as per the FDA guideline. The limit of detection and limit of quantitation for Colchicine was found to be 40.85 pg and 113.01 pg, indicates the sensitivity of the method and was highly reproducible with excellent chromatography properties.

5. CONCLUSION

Proposed study describes new UPLC-MS/MS method for the estimation of Colchicine in human plasma. The validated method exhibit excellent performance in term of selectivity, sensitivity, linearity, accuracy, precision, recovery stability, detection limit and quantitation limit. In addition, the reported method has a short analysis run time. Therefore the proposed method can be used for analysis of estimation of Colchicine to a clinical pharmacokinetic study in human volunteers.

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