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RESEARCH ARTICLE

Sensitive method for the Determination of Colchicine in human plasma by gradient UPLC-ESI-MS/MS: separation of endogenous Interference

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ABSTRACT:
The objective of the current research work was to develop a sensitive, specific, accurate and precise method for the determination of Colchicine in human plasma using Liquid Chromatography Electron Spray Ionization-Tandem Mass Spectrometry. Extraction was done using solid phase extraction technique. Ammonium Acetate 10mM with Acetonitrile was used as mobile phase with gradient programme of 6 minutes on Innoval, C18, 125mm x 4.6mm, 5µ column. Detection was performed on ESI positive mode using MRM transitions 400.400/358.300 for colchicine and 406.400/362.000 for colchicine D6 (ISTD) on API 4000 coupled with Shimazdu Nexara UPLC. Developed method was found to be selective, specific and the Calibration Curve was found to be linear over the range 0.075 to 10.091ng/mL. Endogenous interferences were separated from the peak of interest. Method validation was conducted as per EMEA guideline and the developed method can be applied to Pharmacokinetic/Bioequivalence studies.

KEYWORDS: LC-MS/MS method, Human plasma, gradient elution.

INTRODUCTION:
Colchicine is a medication most commonly used to treat gout. It is a toxic natural product and secondary metabolite. Its molecular formula is C22H25NO6 with molecular weight 399.437. Colchicine is an alternative for those unable to tolerate NSAIDs in gout. At high doses, side effects (primarily gastrointestinal upset) limit its use. At lower doses, which are still effective, it is well tolerated. It is also used as an anti-inflammatory agent for long-term treatment of Behçet's disease. It appears to have limited effect in relapsing polychondritis, as it may only be useful for the treatment of chondritis and mild skin symptoms. Colchicine is used for constipation-predominant irritable bowel syndrome in women, and for treatment of severe or persistent aphthous stomatitis (canker sores).

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Colchicine is also used in addition to other therapy in the treatment of pericarditis. The anti-inflammatory property of colchicine has been used to reduce the recurrence of atrial fibrillation following ablation of cardiac tissue. Colchicine poisoning has been compared to arsenic poisoning. Symptoms start 2 to 5 hours after the toxic dose has been ingested and include burning in the mouth and throat, fever, vomiting, diarrhea, abdominal pain, and kidney failure. These symptoms may set in as many as 24 hours after exposure. Onset of multiple-system organ failure may occur within 24 to 72 hours. This includes hypovolemic shock due to extreme vascular damage and fluid loss through the gastrointestinal tract, which may cause death. In addition, sufferers may experience kidney damage that causes low urine output and bloody urine, low white blood cell counts (persisting for several days), anemia, muscular weakness, and respiratory failure. Recovery may begin within six to eight days. No specific antidote for colchicine is known, though various treatments exist. Certain common inhibitors of CYP3A4 and/or P-gp, including grapefruit juice, may increase the risk of colchicine toxicity. Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to

mitosis, so colchicine effectively functions as a "mitotic poison" or spindle poison. Oral colchicine had been used for many years as an unapproved drug with no prescribing information, dosage recommendations, or drug interaction warnings approved by the U.S. Food and Drug Administration (FDA). Marketing exclusivity has been awarded, As a drug antedating the FDA, colchicine was sold in the United States for many years without having been reviewed by the FDA for safety and efficacy. In 2009, the FDA reviewed a New Drug Application submitted by URL Pharma. They approved colchicine for gout flares, awarding Colcrys a three-year term of market exclusivity, prohibiting generic sales, and increasing the price of the drug from $0.09 to $4.85 per tablet. Several experiments show that the biosynthesis of colchicine involves the amino acids phenylalanine and tyrosine as precursors.[1]

Pharma companies are very interested to enter into the market with colchicine generics. Bioanalysis is integral part of ANDA so reliable, sensitive method is most required. Hence there is a need to develop a sensitive and reproducible method to give services to generic pharma companies.

Literature review indicated many LC-MS methods have been reported by researchers and are available in public domain. However, a detailed review of these papers, indicated that these methods have many disadvantages as explained in the below table:

Table 1: Comparison of methods

Description	**Method** Abe E et al[2]	**Method** Jiang Y et al[3]	**Method** Chen QH et al[4]	**Method** Jarvie D et al[5]	**Method** FENG Yi[6]	**Method** 50238459[7]	**Current method**
Matrix	Plasma or blood	Plasma	Mouse Plasma	Plasma	Plasma	Serum	Plasma
ISTD	embutramide#	tegafur#	berberine#	-	scopolamine#	Codeine#	**Colchicine D6**
System	LCMSMS	LCMSMS	HPLC	HPLC	LCMSMS	HPLC	LCMSMS
Mass technique	ION TRAP~	Triple Quad	-	-	Triple Quad	-	Triple Quad
Detection	MRM,M/Z	MRM,M/Z	UV	UV	MRM,M/Z	UV	MRM,M/Z
Extraction	LLE *	LLE^	PPT##	-	SPE	PPT##	SPE
Run time	5	2.5	12	Rapid	2.5 min	10	6
Flow	Isocratic$	Isocratic$	Isocratic$	-	Isocratic$	Isocratic$	**Gradient**
LLOQ (ng/mL)	2**	0.05	1.5**	-	0.1	2.5ng/µL	0.075
Application	Case report	PK	PK	fatal case	PK	-	PK
Analyte/IS similarity	No	No	No	-	No	No	**Yes**
Sample volume	1 mL	-	-	-	-	-	0.5 mL
Disadvantages of existing methods	*LLE with DCM very complex during separation						
	^LLE with hexane: DCM: IPA combination, small proportion change will impact reproducibility greatly						
	#Analog ISTD, reproducibility less compare to labeled ISTD						
	~Many companies may not have Ion trap systems						
	$Isocratic flow, interferences may not separated out as colchicine is natural product, matrix factor high						
	**No sufficient enough to quantify concentrations of 0.2mg dose colchicine						
	##Protein precipitation is not best over any kind of separation technique						
Advantages of current method	Labeled Internal standard, so greatest reproducibility						
	Gradient column elution, endogenous interferences were well resolved from the analyte of interest						
	LOQ of 0.075 ng/mL which is sensitive enough, lower than other methods except Jiang Y et al method						
	High sensitivity with Low sample volume						
	Only one disadvantage is that of 6.0min run time which is required for gradient to work efficiently						

During our method development, we have faced problems with endogenous interferences however those were successfully overcome using gradient mobile phase. The second problem we have faced is to get Low LOQ with low sample volume, so we have eluted the samples with only 150µL whereas matrix effects were normalized using labeled internal standard. We have not compromised on run time to eliminate endogenous interferences completely however with this method 150samples can be analyzed per day so the turnaround time is low compared to many other methods.

In the current study, we reported a selective, accurate, precise and stable Gradient LC-MS/MS method for the determination of Colchicine in human plasma. The method is suitable for PK sample analysis.

MATERIALS AND METHODS:

Chemicals and Reagents:

Certified pure Working Reference standards of Colchicine and Colchicine D6 were used to prepare required stock/working solutions, JT Baker Methanol and Acetonitrile of HPLC grade and water from in-house Milli-Q system were used for chromatographic purpose, StrataX, 33µm 30mg/mL Cartridges were procured from Phenominex.

Chromatographic and MRM Conditions:

The samples were acquired on the API 4000 MS/MS Coupled with Shimazdu UPLC while the separation was achieved on Innoval, C18, 125mm x 4.6mm, 5µ column using mobile phase consisting of 10mM Ammonium

Acetate and Acetonitrile in gradient method with a flow of 1.0mL/min. Injection volume was 30µL.

The mass instrument was operated in the positive ion mode with unit resolution. Source parameters IS set at 5500, Temperature set at 500^{0}C; DP at 105V and the CE at 30V.

Preparation of Stock and Working Solutions:
1mg/mL Stock solution for Colchicine and Colchicine D6 were prepared by weighting respective working standard and dissolved in methanol. These stocks were stored at 2-8^{0}C. Working spiking solutions of Colchicine were prepared in 80% methanol in water.

Sample Extraction Procedure:
To an aliquot of 0.5mL plasma sample in RIA vial, 50 µL of Internal standard working solution was added and vortexed; 200µL of 0.02% Formic Acid was added and then vortexed; and then samples were subjected to Solid phase extraction using Strata X 1cc/30mg cartridges. Cartridges were conditioned with methanol and followed by equilibration with 0.02% Formic Acid. Preprepared samples then loaded onto equilibrated cartridges and passed slowly. Cartridges were washed with water and 0.02% Formic Acid; eluted with 0.150mL Elution solution (10mM Ammonium formate: Acetonitrile; 10:90) and then transferred to auto-sampler vials for analysis.

Preparation Of Calibration Curve Standards And Quality Control Samples:
Eight Calibration curve standards were prepared in bulk for the calibration curve range by spiking 2% of the respective standard working spiking solution in screened K_3EDTA blank plasma. Four levels of quality controls were also prepared from their respective working spiking solutions along with calibration curve standards. All spiked samples were aliquoted and were stored at -70^{0}c.

RESULTS AND DISCUSSION:

Specificity:
Eight (08) lots of Human Plasma (06 Normal Human Plasma lots, 01 Haemolysed Human Plasma and 01 Lipemic Human Plasma) containing K_3EDTA as an anticoagulant were evaluated for their selectivity. No significant interference was observed at the retention time of analyte and internal standard in all the 08 evaluated lots when comparing the blank matrix response against LLOQ sample. Acceptance criteria is, The percentage interference should be ≤ 20.00% of area of LLOQ for analyte and should be ≤5.00% of area of ISTD observed in LLOQ sample for ISTD. At least 80.00% lots should meet the acceptance criteria. Sample chromatogram presented as Fig 1 and Fig 2.

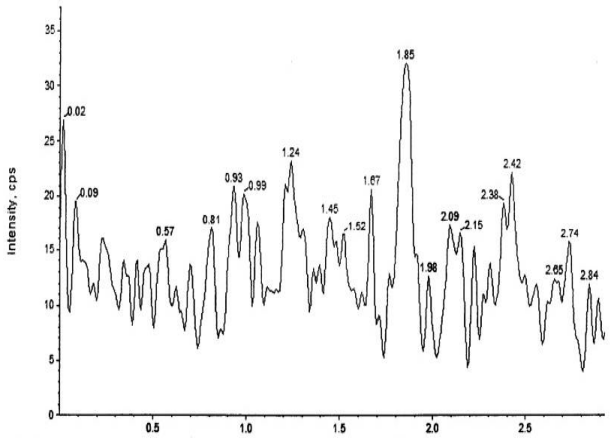

Fig.1: Blank sample chromatogram

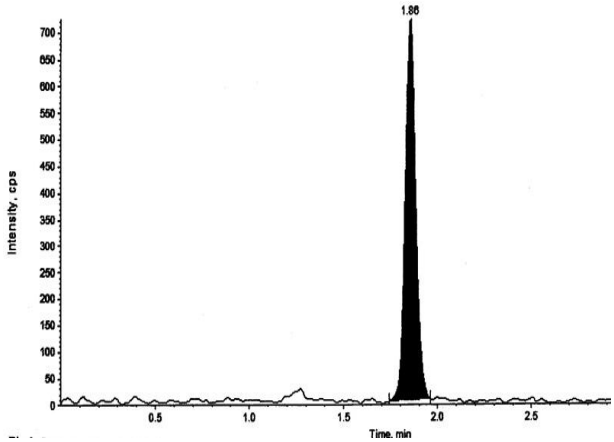

Fig.2: LLOQ sample chromatogram

Selectivity in Presence of the Concomitant Drugs:
Selectivity of Colchicine was determined in presence of co-administered drugs Caffeine Anhydrous, Cetirizine Dihydrochloride, Nicotine Tartrate, Ibuprofen and Pantaprazole Magnesium. Six blank samples containing co-administered drugs having concentrations 10347.502 ng/mL for Caffeine Anhydrous, 448.623ng/mL for Cetirizine Dihydrochloride, 154.609ng/mL for Nicotine Tartrate, 20416.974ng/mL for Ibuprofen and 12454.671 for Pantaprazole Magnesium. Six samples containing above mentioned drugs and Colchicine at LLOQ (0.500 ng/mL) concentration were analyzed together. No significant interference was observed at the retention time of Analyte (Colchicine) and ISTD (Colchicine D6) and Six out of six LLOQ samples of Colchicine met the acceptance criteria. No significant interference was observed at the retention time or ISTD (Colchicine D6) in Blank + Analyte samples.

Linearity, Precision and Accuracy including Ruggedness for column and analyst change:
A linear equation was established to produce the best fit for the concentration vs. area response relationship. The regression type was 1/(concentration)^2 and peak area

ratio for a 08-point calibration curve was found to be linear from 0.075ng/mL to 10.091ng/mL for Colchicine. The goodness of fit (r^2) was consistently greater than 0.98 during the course of validation. The range of precision and accuracy of the back-calculated concentrations of four standard curve was from 0.70% to 5.03% and from 96.65% to 105.96% respectively for Colchicine. Intra day precision ranged from 0.96% to 17.24% whereas inter day precision ranged from 1.94% to 6.91%. Intra day accuracy ranged from 87.79% to 103.36% whereas inter day accuracy found 93.57% to 99.48%. Sample linearity presented as Fig 3.

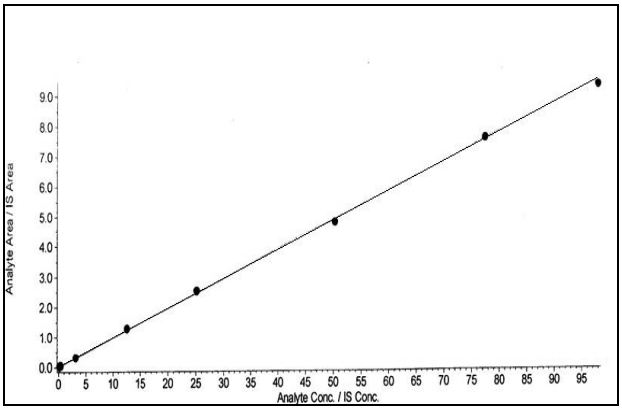

Fig.3: Sample calibration curve

Limit of Quantification (LOQ) Determination

Limit of Quantification were determined during P and A Batch 03 by processing six samples of each LLOQQC (Limit of Quantification Quality Control) from P and A Batch 03 and 06 ULOQ samples. The precision of LLOQQC and ULOQ samples was 12.99% and 3.83% respectively for Colchicine which was within the acceptance criteria of ≤20.00% for LLOQQC and ≤ 15.00% for ULOQ. The accuracy of LLOQQC and ULOQ samples was 102.00% and 92.56% respectively for Colchicine which was within the acceptance criteria of ± 20.00% for LLOQQC and ± 15.00% for ULOQ.

Extraction Recovery:

The recovery was determined by comparing the detector response of Colchicine and Colchicine D6 at three distinct levels of extracted low, middle and high quality control samples of Recovery 02 with detector response obtained from the aqueous quality control samples. The % recovery for the lower, middle and higher quality control samples was 37.23%, 36.92% and 36.84% respectively and global recovery for Colchicine was 36.996 and 37.25% at all three level for Colchicine D6. The %CV of mean global recovery at lower, middle and higher quality control was 0.55% for Colchicine which was within the acceptance criteria of ≤20.00%.

Matrix Effect:

Matrix effect was evaluated by processing six matrix lots including one hemolysed and one lipemic human plasma lot as per STP. These blank samples were spiked at LQC and HQC level and compared with equivalent aqueous low and high quality control samples. The area ratios of both aqueous and spiked sample were compared. Then matrix factor and IS normalized Matrix factor was calculated. The precision of IS normalized Matrix factor at low and high quality control samples were 4.69% and 2.40% respectively for Colchicine which was within the acceptance criteria of ≤15.00%.

Other Experiments:

Batch size evaluation, Haemolysis and Lipemic effect, Dilution Integrity, Reinjection reproducibility were also evaluated and found acceptable.

Stability Evaluation:

Stabilities including short term stock/working solution, long term stock/working solution, auto-sampler, bench top, wet extract, wet extract bench top, Freeze thaw, Whole blood, long term in plasma were conducted and found no stability issues.

Table 2: Stability Details:

Type of Stability	Condition	Duration
Autosampler Stability	5.0°C	27 hours and 41 minutes
Wet Extract Stability	2.0°C to 8.0°C	22 Hours and 51 minutes
Bench Top Stability	Ambient	06 hours and 18 minutes
Wet Extract Bench Top Stability	Ambient	07 hours and 16 minutes
Freeze Thaw Stability	-20.0^{0}C ±5.0^{0}C	4 cycles
Whole Blood Stability	Ambient	02 hours and 12 minutes
Short Term Stock Solution Stability	Ambient	08 Hours and 08 minutes
Long Term Stock Solution Stability	2.0°C to 8.0°C	10 days and 07 Hours

CONCLUSION:

A LC-MS/MS method for the determination of Colchicine in Human Plasma using Innoval C18 ,4.6 x 125mm, 5µ column over the CC range of 0.075ng/mL to 10.091ng/mL has been developed and met the validation requirements. Stability was demonstrated for Colchicine and Colchicine D6 in aqueous and Human Plasma samples under varying conditions of storage and found to give satisfactory results. The method can be applied to PK/Bioequivalence studies.

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CONFLICT OF INTERESTS:

The authors declare no conflict of interest.

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