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DETERMINATION OF COLCHICINE IN HUMAN PLASMA BY A SENSITIVE LC-MS/MS ASSAY

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ABSTRACT

Colchicine is natural product commonly used to treat gout. The present paper describes the method development and validation of colchicine in human plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Colchicine-d6 was used as an internal standard (IS). Sample extraction was carried out using simple solid phase extraction (SPE). The extracted samples were chromatographed on C_{18} column using isocratic mobile phase composed of 10 mM ammonium formate (pH 3.5) and methanol 20:80, v/v. The method was linear over the range of 0.04-10.0 ng/mL. A total of five calibration curves were generated during the validation to calculate precision and accuracy. The stability of analyte was extensively evaluated in plasma as well as in aqueous samples and results were met the acceptance criteria defined in US FDA guidelines.

KEYWORDS: Colchicine; Solid–phase extraction (SPE); LC–MS/MS; Method validation.

INTRODUCTION

Gout is a condition where uric acid levels in the blood are too high and may forms uric acid crystals in the joints. This leads to inflammatory arthritis characterized by recurrent attacks of a

red, tender, and swollen joint. Colchicine is natural product mainly used to treat gout. It is extracted from the plant *Colchicum autumnale*. Colchicine works by decreasing swelling and lessening the buildup of uric acid crystals that cause pain in the affected joint(s).[1] Recently, the drug is also being used for the treatment of other neutrophilic disorders such as familial mediterranean fever (FMF) and Behçet disease (BD)[2] Colchicine is absorbed by the jejunum and ileum and is readily bioavailable after oral administration. Absolute bioavailability was reported to be approximately 45%.[3]

Literature survey reveals that many LC-MS/MS have been reported for the determination of colchicine in a variety of biological samples like human serum,[4] human plasma[5,6] and whole blood.[7, 8] Recently, the author Fabresse *et al.*, 2017[9] reported a LC-MS/MS method for the quantitation of free and fab-bound colchicine in plasma, urine and organs in minipigs. Gul *et al.*, 2015[4] describes a method for the determination of colchicine in human serum samples using pimozide as internal standard. The linearity range was 1.5-25 ng/mL, which is narrow range not suitable for routine drug analysis in plasma for pharmacokinetic study. Abe *et al.*, 2006[5] published an LC-MS/MS method for the determination of colchicine in 1 mL of human plasma using liquid-liquid extraction. Embutramide used as an internal standard. Bourgogne *et al.*, 2013[6] reported a turbulent-flow LC-MS/MS for colchicine determination in plasma using protein precipitation method for sample extraction. Calibration curves ranged from 0.342 to17.1 ng/mL and the total run time was 9.5 min which is very high and not suitable for high through put bioanalysis. Jones *et al.*, 2002[7] and Wehner *et al.*, 2006[8] analyzed colchicine in whole blood using LC-MS/MS method with run time >5 min. Moreover, Wehner *et al.*, 2006[9] employs more plasma volume (1 mL) for sample preparation. An efficient bioanalytical method should rapid, simple and consume less sample volume for analysis. Also, it should be specific and selective to avoid possible inferences at mass transition of analyte and the internal standard.[10,11] The reported methods were suffering from lack of sensitivity, use of more sample volume, longer chromatographic run time and employs non-deuterated compounds as internal standards which may results poor precision and accuracy values, where compensation for matrix effect is not possible.

In view of above, we have developed and validated a simple, reliable and rapid LC-MS/MS method for the determination colchicine in human plasma. The present method is having run time of 4 min and utilized very low plasma 200 µL for sample preparation. Also, the sample extraction method with cartridges was efficient in obtaining high recovery for analytes with

no or minimal matrix effect. Here we used colchicine-d6 as internal standard.

EXPERIMENTAL

Standards and reagents

A 93.3% pure standard of colchicine was obtained from Indoco Remedies Limited, whereas 99.9% pure standard of colchicine-d6 (Fig.1) was obtained from Clearsynth Labs Limited (Mumbai, India). LC-MS grade methanol was obtained from J.T. Baker (Phillipsburg, USA). Analytical grade ammonium formate and formic acid were procured from Merck Ltd (Mumbai, India). Blank human plasma was obtained from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions

A Shimadzu (Kyoto, Japan) HPLC system coupled with an AB Sciex (Foster City, CA, USA) API-4500 triple quadrupole mass spectrometer was used for the study. An aliquot 15 µL of the samples was injected on to Synergy™ 4.0 µm Polar-RP 80Å (75 x 4.6 mm) column using an isocratic mobile phase of 10 mM ammonium formate (pH 3.5) and methanol (20:80, v/v), delivered at a flow rate of 0.5 mL/min. The mass spectrometer was operated with ESI probe in positive mode at a source temperature of 500 °C and ion spray voltage of 5000 V. The source parameters namely curtain gas, collision gas nebulizer gas (GS1) and auxiliary gas (GS2), were set at 40, 9, 40, and 40 psi, respectively. The compound parameters like the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and entrance potential were 60, 30, 13, 10 V for colchicine and for the IS. Ions were monitored in the multiple–reaction monitoring (MRM) mode with the transition pairs of *m/z* 400.3 precursor ion to the *m/z* 358.3 for colchicine and *m/z* 406.3 precursor ion to the *m/z* 362.2 product ion for the IS. The chromatographic data was processed by Analyst Software™ (version 1.6.1).

Stock solutions

Two separate stock solutions were used to prepare the calibration (CC) and quality control spiking solutions. The stocks were prepared in methanol, whereas further dilutions were made in diluent (water and methanol, 30:70, v/v). Calibrates were prepared in plasma at a concentration levels of 0.04, 0.08, 0.20, 0.40, 1.00, 2.00, 4.00, 6.01, 8.01 and 10.0 ng/mL. Similarly, the quality control (QC) samples were prepared at concentrations of 0.04 (lower limit of quantitation, LLOQ), 0.12 (low quality control, LQC), 1.51 (medium quality control,

MQC1), 5.02 (MQC2) and 7.52 ng/mL (high quality control, HQC). Samples were prepared in bulk and stored at -70±10 °C in a freezer. Similarly, the IS stock solution was prepared in methanol and further diluted to 50 ng/mL with diluent.

Sample preparation

All the samples were allowed to equilibrate to room temperature. An aliquot of 200 µL of plasma sample was spiked in to 2 mL polypropylene tubes. To each sample, 25 µL of the IS dilution (50 ng/mL of colchicine-d6) was added. Then, added 250 µL of water and vortex mixing for 10 s. The sample mixture was loaded onto a Strata^{TM} X 33µm polymeric sorbent (30 mg/1 mL) that was pre–conditioned with 1.0 mL of methanol followed by 1.0 mL of water. The extraction cartridge was washed with 1.0 mL of water and eluted with 0.5 mL of mobile phase and injected into the LC-MS/MS system.

Method validation

The developed method was validated for selectivity, sensitivity, carryover test, matrix effect, linearity, precision and accuracy, recovery, dilution integrity, and stability.[12]

RESULTS AND DISCUSSION

Method development

Mass spectrometer was operated using ESI source in multiple reaction monitoring mode (MRM). Mass spectrometer parameters were optimized by infusing (5 µL/min) the 50 ng/mL concentration of analyte and the IS separately. High intense peaks were found in positive mode than the negative mode. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from m/z 400.3 to 358.3 for colchicine and from m/z 406.3 to 362.2 for the IS. The dwell time for each transition was 200 ms. As per the reports MRM technique provides inherent selectivity and sensitivity, hence we applied for the present study.[13-17]

Mobile phase composition was optimized with acetonitrile and methanol in combination with acidic buffers ammonium formate and ammonium acetate and volatile acids namely formic acid and acetic acid. Also, a variety of C_{18} (Synergy Polar-RP, Zodiac C_{18}, Zorbax SB C_{18}, Hypersil BDS, Zorbax SB C_8, Inertsil ODS, Alltima HP C_{18}, Ace 5 C_{18}, Grace, Kromasi etc.) columns were tested to get symmetric peak shape with short retention time. Analyte response was not reproducible with ammonium formate and ammonium acetate without adjusting the

pH in combination with methanol or acetonitrile. Hence, the pH of ammonium formate buffer was adjusted to 3.5 with formic acid. Among different mobile phase combinations tested, 10 mM ammonium formate (pH 3.5, adjusted with formic acid) and methanol (20:80, v/v) gives symmetric peak shape and reproducible response for the analyte. Best chromatography results were obtained with Synergy™ 4.0 µm Polar-RP 80Å (75 x 4.6 mm). The run time was set at 4 min with retention time of 2.9 min for colchicine and the IS. Solid phase extraction (SPE) with Strata™ X 33µm polymeric sorbent (30 mg/1 mL) cartridges were used to extract the analyte from plasma, which gives neat and clear extract with minimal or no matrix effect. Colchicine stable labeled isotope colchicine-d6 was used as internal standard which exerts similar extraction, ionization and retention time with analyte.

Carryover test

To check the effect of carryover of analyte and the IS in subsequent runs, a blank sample was injected after the highest concentration of analyte (ULOQ) and working concentration of the IS. No significant carryover was observed in the blank samples after injection of ULOQ along with the IS.

Sensitivity

Sensitivity can be defined as lowest limit of reliable quantification (LLOQ), which is set at 0.04 ng/mL for the present method. Six replicates of LLOQ samples were analyzed with standard CC. The precision and accuracy LLOQ concentration was 2.42 and 107%, respectively. Also, the signal–to–noise ratio (S/N) measured was ≥10.

Selectivity and chromatography

K3 EDTA human plasma lots from six different source were screened for possible interferences. These includes one lipemic and one haemolyzed plasma. Fig. 2 shows that, no significant interference in the blank plasma at the retention time of analyte and the IS. Also, no interference was observed from the IS channel at the retention time of analyte. A representative chromatogram LLOQ samples was displayed in Fig. 3.

Specificity

Cross talk or interference was assessed by injecting the analyte and the IS separately. The analyte was injected at ULOQ concentration and the IS was injected at working concentration. Results shows that there was no significant cross talk was observed.

Matrix effect

Matrix effect was determined at LQC and HQC levels. It was expressed as IS normalized matrix factor (MF). The response of post–extraction spiked samples was compared with mean area of neat samples. The IS normalized matrix factor was 0.99 for LQC and 1.00 for HQC. The results show no substantial matrix effect was found in all the plasma lots tested.

Linearity, precision and accuracy

A total of five successful calibration curves run during the validation in the concentration range of 0.04–10.0 ng/mL for colchicine. The mean correlation coefficient was ≥ 0.99. A regression equation with weighting model $1/x^2$ of the drug to the IS was found to be best fit for the concentration–detector response relationship. The results of intra–day and inter–day analysis are summarized in Table 1. The results revealed good precision and accuracy.

Recovery

The recoveries was determined at LQC, MQC2 and HQC levels and were found to be 95.9%, 98.5% and 97.4%, respectively with mean recovery of 97.3±1.30%. Similarly, the recovery of IS was 98.8% with the precision range of 0.40–1.33%.

Dilution integrity

The ULOQ can be extended up to 20.0 ng/mL by 1/2 and 1/4 dilutions with screened human blank plasma. The precision (%CV) and accuracy for two-fold dilution was 0.71% to 98.4%, respectively. Similarly, the precision (%CV) and accuracy for four-fold dilution was 0.55% to 98.5%, respectively.

Stability studies

The mean % nominal values were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels and the precision (% CV) values were within 15% (Table 2) for all the stability tests carried out during the entire course of method validation. All the above stability results were found to be within the acceptable limits during the entire validation.

Table 1: Precision and accuracy data for colchicine.

Quality control Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day (*n*=12)			
LLOQ	0.04 ± 0.004	9.31	101
LQC	0.12 ± 0.005	3.64	103
MQC1	1.52 ± 0.02	1.62	101
MQC2	5.15 ± 0.05	1.06	103
HQC	7.67 ± 0.09	1.23	102
Inter–day (*n*=30)			
LLOQ	0.04 ± 0.003	8.22	104
LQC	0.12 ± 0.004	3.85	102
MQC1	1.51 ± 0.08	1.46	101
MQC2	5.12 ± 0.08	1.62	102
HQC	7.58 ± 0.16	2.08	101
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 0.04, 0.12, 1.51, 5.02 and 7.52 ng/mL, respectively.			

Table 2: Stability data for colchicine in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Auto-sampler (75 h)	0.12	0.12 ± 0.004	2.83	103
	7.52	7.58 ± 0.04	0.47	101
Wet extract stability (69 h)	0.12	0.12 ± 0.004	3.11	103
	7.52	7.68 ± 0.05	0.61	102
Bench top (13 h)	0.12	0.12 ± 0.004	3.39	102
	7.52	7.66 ± 0.04	0.57	102
freeze and thaw (4 Cycles)	0.12	0.12 ± 0.004	3.57	101
	7.52	7.64± 0.05	0.67	102
Re-injection (27 h)	0.12	0.13 ± 0.006	4.54	106
	7.52	7.69 ± 0.04	0.52	102
Long–term (62 days)	0.12	0.12 ± 0.005	3.84	101
	7.52	7.67 ± 0.07	0.96	102

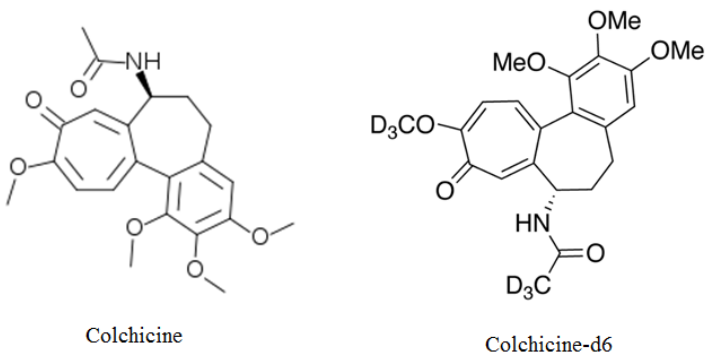

Figure 1: Chemical structure of colchicine and colchicine-d6 (IS).

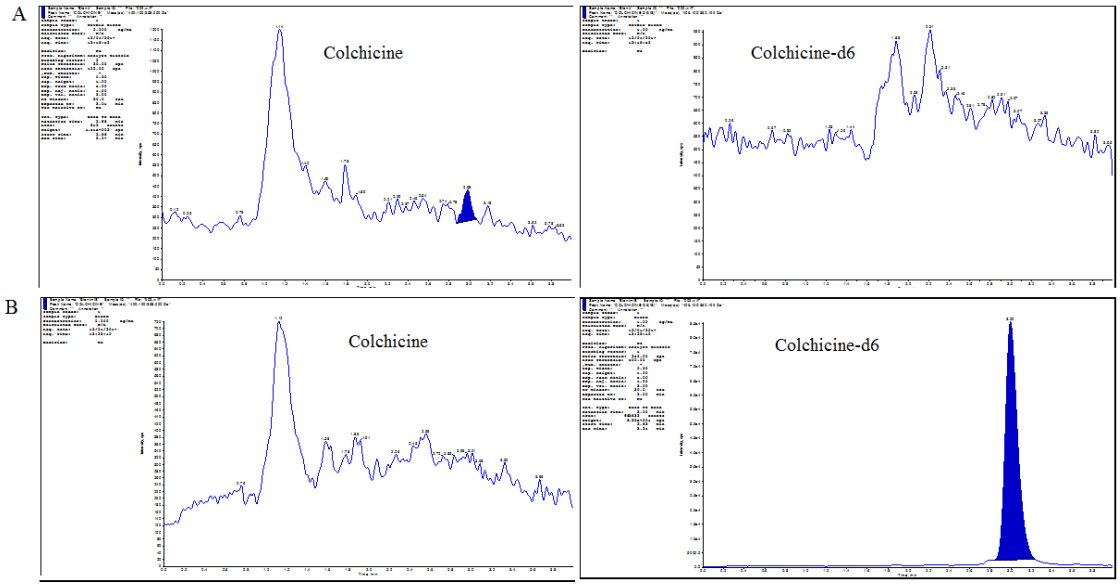

Figure 2: Typical MRM chromatograms of colchicine (left panel) and IS (right panel) (A) extracted blank plasma (B) blank plasma spiked with IS.

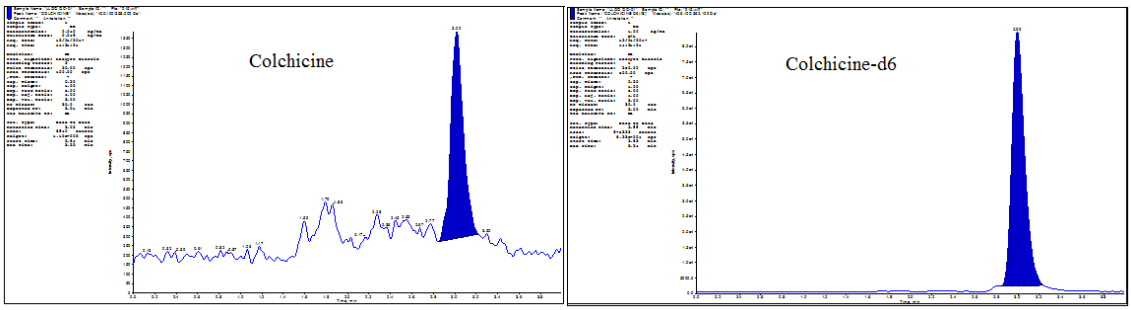

Figure 3: Typical MRM chromatogram of an LLOQ sample of colchicine (left panel) along with IS (right panel).

CONCLUSIONS

In conclusion, the described LC–MS/MS method is simple, sensitive and selective for the quantification of colchicine in human plasma. With the proposed method colchicine can be determined as low as 0.04 ng/mL after post dosing in-vivo samples using low plasma volume (200 µL). Simple and one step SPE extraction give high and reproducible recovery colchicine and the IS. Also, matrix effect was negligible. This method was fully validated as per US FDA guidelines and is well suitable for pharmacokinetic or bioavailability/bioequivalence application.

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