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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY – MASS SPECTROMETRIC BIOANALYTICAL METHODFOR DETERMINATION OF THE COLCHICINE IN HUMAN PLASMA FOR BIOEQUIVALENCE STUDY APPLICATION

Eyad Mallah[1*], Razan Bardees[1], Luay Abu Qatouseh[1], Kamal Sweidan[2], Basel Arafat[3], Da'san Jaradat[4], Tawfiq Arafat[5]

[1]Faculty ofPharmacy and Medical Sciences, University of Petra, Amman –Jordan.
[2]Department of Chemistry, University of Jordan, Amman –Jordan.
[3] Faculty of Health, Education, Medicine and Social Care, Bishops Hall Lane, Chelmsford, UK.
[4]Department of Chemistry, Faculty of Science, Al-Balqa Applied University, Al-Salt, Jordan
[5]Jordan Center for Pharmaceutical Research, Amman –Jordan.

Abstract— Colchicine is a medication used to treat gout and Behçet's disease. A rapid, stable and sensitive reversed phase liquid chromatography method coupled with MS/MS detector was developed and validated for determination of Colchicine in human plasma, Colchicine and internal standard (Colchicine-D_3) were usefully extracted from human plasma samples by using liquid-liquid extraction technique.Validation parameter was carried out for the chromatographic method used for determining Colchicine in plasma including: accuracy, precision, linearity, selectivity, stability, calibration curve, recovery. The mobile phase consisting of (85% methanol: 15% Ammonium chloride), column ACE C_8, (50 X 2.1) mm, 5 µm, flow rate was 0.4 ml/min, retention time was 0.44 minute for colchicine and Colchicine- D_3, and the total run time was 0.8 minute. The lower limit of quantitation was 0.05 ng/mL, the calibration curve was linear (R^2 = 0.9985) over the range of (0.05 – 4.00) ng/ml.The intra- and inter day precisions for quality control samples were < 10.0%, and the intra- and inter day accuracies were in the range of (89.33-106.33) %. However, the current LC/MS method of colchicine could be applied for pharmacokinetic study in human plasma.

Keywords: Colchicine, pharmacokinetic, LC/MS, human plasma.

1. Introduction:

colchicine (figure 1) has been extracted from a plant called (Colchicum (autumn crocus)[1], it regards a tricyclic alkaloid medication which has a lipophilic property[2] and classified as a narrow therapeuticwindow drug[3]. Colchicine is an approved drug by the FDA for 'gout flares' and familial Mediterranean Fever[4]. For many years colchicine has been used within the treatment plan of many different diseases such as: pericarditis, many trials shows its effect in reducing the risk of cardiovascular event for patient already suffering from cardiac issues[5], in the last few years it become also used for some dermatological diseases[3]. It emerges as a thinkable new, helpful, safe and inexpensive drug for the treatment of coronary syndrome (both acute and chronic)[2].There are many different methods of administration of colchicine; for exampleit has been used orally or topically for different indications in dermatological diseases[3].Colchicine may have unserious side effects such as : nausea vomiting and diarrhea[6].

Colchicine works at the cellular level, it mainly affect the cell splitting[7], it showed up its effect by the obstruction of polymerization process of themicrotubules; the part that has a major role in many critical operations in the cell, like: cell splitting, gene expression and other processes.This part composed of non-identical dimers (alpha and beta). colchicinestarts the task of preventing the polymerization process by binding to these dimers. colchicine and tubulin builds up a complex that restrict the elongation process so that cell splitting cannot be completed anymore[3]. Furthermore, colchicine accumulates in white

blood cells “leucocytes” in concentration more that plasma affecting on it and causes disruption in cell splitting[3, 8].

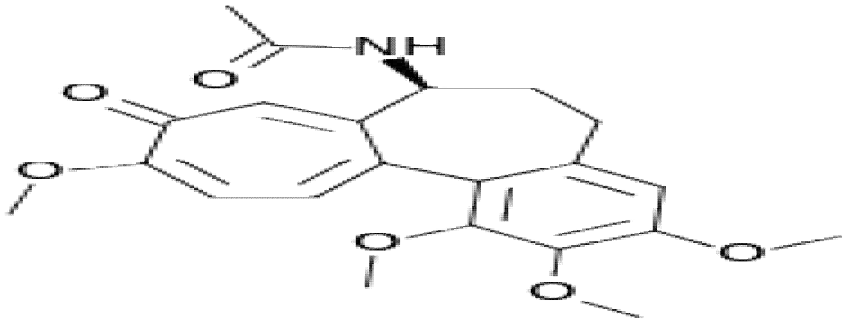

Fig 1: Colchicine Chemical Structure[9]

Colchicine can be directly determinate in pharmaceutical and plasma“biological fluid" by rapid HPLC technique. Taylor et. al. used a reversed phase -high performance liquid chromatographic for the determination of colchicine[10].It is well known that HPLC–MS further enhances specificity, so based upon that E. Abe *et al* developed liquid chromatography coupled to ion trap mass spectrometry (MS) detection with electrospray ionization interface for the quantification of colchicine in human plasma, they used liquid –liquid extraction technique for the isolation of colchicine from plasma, the lower limit of quantitation was 0.5 ng/ml[11].Furthermore, liquid chromatography – tandem mass spectrometry method was also used for the determination of colchicine in human plasma over the range of (0.050-10ng/ml) [12].

The current study describes a new, simple, highly sensitive and selective LC–MS analytical method based on liquid- liquid extraction of colchicine in human plasma.

2. EXPERIMENTAL SECTION

2.1. Reagents and chemicals

Ammonium chloride (extra pure), de-ionized water, methanol (HPLC grade), Methyl tert-butyl ether, dichloromethanewere purchased from Fisher scientific. Human Plasma, Colchicine and Colchicine D3 raw materials were obtained fromAthos Chemicals and Chemsky (shanghai) International Co.Ltd**,** respectively.

2.2. Instrumentation

API 4000 Mass Spectrometer was used and composed of a constant auto Sampler (Agilent 1200), ACE column C_8, (50 X 2.1) mm, 5 µm. 100 µL fixed injector (Agilent 1200), on-line vacuum. Degasser (Agilent 1200).

2.3 Preparation of stock solutions

2.3.1 Colchicinestock Standard Solution (A)

10mg of Colchicine working standard was dissolved in 10ml methanol to get a concentration (1.0mg Colchicine /ml) stock solution (A).

2.3.2 Colchicine- D_3(I.S)stock Solution(B)

The Colchicine- D_3vial’s content dissolved with equivalent volume of MeOH to get a concentration (1.0 mg Colchicine- D_3/mL).

2.4 Preparation of working Standard Solutions

2.4.1 ColchicineWorking Solution(A-1, A-2)

A 100 µL of Colchicine stock solution (A) (1.0 mg/mL) was diluted into 10.0 mL of (80:20%; v/v) methanol: water. The obtained concentration was (10.0 µgColchicine/mL) (working solution A-1).After that, 200 µL of Colchicine working solution (A-1) diluted into 10.0 mL of (80:20%; v/v) methanol: water. The obtained concentration was (0.2 µgColchicine/mL) (working solution A-2).

2.4.2 Colchicine- D_3(I.S) Working Solution

A 50 µL of Colchicine- D_3stock solution (B) (1.0 mg/mL) was diluted into 10.0 mL of (80:20%; v/v) methanol: water. The obtained concentration was (5.0 µgColchicine- D_3 /mL) (working solution B-1). After that, 50 µL of Colchicine- D_3 working solution (B-1) diluted into 50.0 mL of (80:20%; v/v) methanol: water. The resultant concentration was (5.0 ng Colchicine- D_3 /mL) (working solution B-2).

2. 5 Buffer Solution for Mobile phase (0.5mM Ammonium chloride)

A 0.01337g of Ammonium chloride dissolved in 500.0 mL of de-ionized water to get a concentration of 0.5mM.

2.6 Calibration Standards & Quality Control Samples Preparation

Calibration standards and quality control samples (QC) for this study were prepared by using spiking human plasma with working solutions containing Colchicineas shown in tables (2.1A, 2.1 B, 2.2 A and 2.2 B).Spiked plasma samples were stored at -40 C.

Table 2.1A: Calibration Standards Preparation in diluent(80:20%; v/v) methanol: water

(Serial Solutions of Colchicine)				
Solution No:	**Working Solution [(1)] Concentration (ng/mL)**	**Stock/working Concentration (ng/mL)**	**Volume Taken from Stock (µL)**	**Total Volume (mL)**
S1	0.5	200.0	50.0	20.0
S2	1.0	200.0	50.0	10.0
S3	2.0	200.0	100.0	10.0
S4	4.0	200.0	200.0	10.0
S5	8.0	200.0	400.0	10.0
S6	15.0	200.0	750.0	10.0
S7	25.0	200.0	1250.0	10.0
S8	40.0	200.0	2000.0	10.0

[(1)] Prepared from working solution (A-2)

Table 2.1B: Quality control sample Preparation in diluent (80:20%; v/v) methanol: water

(Serial Solutions of Colchicine)				
Solution No:	**Working Solution [(1)] Concentration (ng/mL)**	**Stock Concentration (ng/mL)**	**Volume taken from stock (µL)**	**Total Volume (mL)**
S9	0.5	200.0	50.0	20.0
S10	1.5	200.0	75.0	10.0
S11	16.0	200.0	800.0	10.0
S12	32.0	200.0	1600.0	10.0

[(1)] Prepared from working solution (A-2)

Standard no.	Working Solution (ng/mL)	Spiked Volume (µL)	Total Plasma Volume (µL)	Calibrator (ng/mL)
Cal 01	0.5	50.0	500.0	0.050
Cal 02	1.0	50.0	500.0	0.100
Cal 03	2.0	50.0	500.0	0.200
Cal 04	4.0	50.0	500.0	0.400
Cal 05	8.0	50.0	500.0	0.800
Cal 06	15.0	50.0	500.0	1.500
Cal 07	25.0	50.0	500.0	2.500
Cal 08	40.0	50.0	500.0	4.000

Table 2.2 A: Calibration Standards Preparation in spiked plasma

Table 2.2 B: Quality control Preparation in spiked plasma

QC no.	Working Solution (ng/mL)	Spiked Volume (µL)	Total Plasma Volume (mL)	Calibrator (ng/mL)
$\mathbf{QC_{LLOQ}}$	0.5	50.0	500.0	0.050
$\mathbf{QC_{Low}}$	1.5	50.0	500.0	0.150
$\mathbf{QC_{Med}}$	16.0	50.0	500.0	1.600
$\mathbf{QC_{High}}$	32.0	50.0	500.0	3.200

2.7 Extraction Procedure

50 mL of internal standard (5.0 ng Colchicine_D_3/ml) was added to 500 mL of spiked/blank plasma samples into a previously labeled test tube, vortex for 15 seconds, after that 5 mL of extraction solvent (20%DCM: 80%MTBE) was added and vortex for 5 minutes, then centrifuge the sample for 5 minutes at 4400 rpm. Freeze the sample for about 30 minutes then decant the supernatant in a clean labeled evaporating glass tube. Evaporate the extraction solvent by compressed air in water bath at 40°C; (this step should be conducted in the fume hood). Reconstitute the residue with 300 mL of reconstitution solution, and vortex for 1 minute. Finally, transfer the sample into a flat bottom insert's vial, and inject into the LC instrument.

2.8 Chromatographic Conditions

The HPLC optimized conditions, MS Parameters, compound's detection and retention times are summarized in table (2.3).

Table 2.3: Chromatographic Conditions

Flow Rate	0.4 ml/min

Positive Mode					
CUR	**CAD**	**GS1**	**GS2**	**Temp.**	**IS Voltage**
20	7	60	40	600	5500

Compound Name	Detection	Retention Time
Colchicine	Parent 399.9 and daughter 358.1	0.44 minute
Colchicine- D_3	Parent 403.1 and daughter 359.2	0.44 minute

Column Temperature	30 °C
Autosampler Temperature	10 °C
Injection Volume	10 µl
Total Run Time	0.8 minute
Column	ACE C_8, (50 X 2.1) mm, 5 µm

Compound Name	DP	EP	CE	CXP
Colchicine	83	9	30.5	20.0
Colchicine- D_3	77	10	31.5	19.5

2.9Colchicinemethod validation

According to the European EMEA guideline, the method was validated in human plasma [13]. The validation was performed to evaluate the method in terms of selectivity, accuracy, precision, sensitivity, calibration curve (linearity of response), recovery and stability.

2.9.1 Selectivity

Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analyzed and evaluated for interference. Use of fewer sources is acceptable in case of rare matrices. Normally, absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the Colchicine and 5% for the Colchicine- D_3 internal standard. [13]

2.9.2 Lower Limit of Quantification

The lower limit of quantification (LLOQ) is the lowest concentration of Colchicine in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ is considered being the lowest calibration standard. In addition, the Colchicine signal of the LLOQ sample should be at least 5 times the signal of a blank sample. The mean concentration for at least five LLOQ samples should be within 20% of the nominal value, and the CV value should not exceed 20% (as per US-FDA 2001 guideline) [14].

2.9.3 Calibration curve and linearity

A minimum of six calibration concentration levels should be used, in addition to the blank sample (processed matrix sample without analyte and without I.S) and a zero sample (processed matrix with I.S).The blank and zero samples should not be taken into consideration to calculate the calibration curve

parameters.The calibration curve parameters should be reported (slope and intercept in case of linear fit). In addition, the back calculated concentrations of the calibration standards should be presented together with the calculated mean accuracy values. All the available (or acceptable) curves obtained during validation, with a minimum of 3 should be reported. [13]

2.9.4Accuracy

The accuracy of the analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the Colchicine (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the Colchicine, the quality control samples (QC samples).The mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value. [13]

2.9.5Precision

The precision of the analytical method describes the closeness of repeated individual measures of Colchicine. Precision is expressed as the coefficient of variation (CV). Precision should be demonstrated for the LLOQ, low, med and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of accuracy."EMEA 2011

The CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.**[13]**

2.9.6 Stability

Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of the analyte. The mean concentration at each level should be within ±15% of the nominal concentration. [13]

A triplicate from LLQ and QC samples were estimated the stability validation sections, and calculated upon freshly spiked calibration curve. long term storage was done under −40◦C. Freeze–thaw cycles stability of the samples has been obtained over five freeze-thaw cycles by thawing from frozen state at room temperature for 1 h and refrozen for 24 h.

2.9.7 Recovery

The recovery of Colchicine is the detector response obtained from an amount of the Colchicine added to and extracted from the plasma, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, med and high) with un-extracted standards that represent 100% recovery. [14]

3. Results and discussion:

3.1 Full Analytical Method Validation of Colchicine

3.1.1 Selectivity

Selectivity test was performed using a batch containing a calibration curve covering the quantification range, six standard blank samples of the matrix from different sources extracted using the proposed extraction procedure, theresults aresummarized in (table 3.1)

Table 3.1: Selectivity of Colchicine in blank plasma samples

Sample Name	**LLOQ Colchicine Area = 2661**	
	Blank Area (Analyte)	**Interfering%**
Plasma (1)	0	0.00
Plasma (2)	271	10.18
Plasma (3)	207	7.78

Sample Name	LLOQ Colchicine Area = 2661	
	Blank Area (Analyte)	Interfering%
Plasma (4)	131	4.92
Plasma (5)	231	8.68
Plasma (6)	182	6.84

The selectivity results show that, there are interferences within acceptance criteria from endogenous plasma samples with Colchicine. [13]

3.1.2 Lower limit of quantification (LLOQ)

For the assay of Colchicine, the lower limit of quantification (LLOQ) was 0.05 ng/mL.
The LLOQ test was established using six samples of the 1st day precision and the results are illustrated in table 3.2.

Table 3.2: Lower Limit of Quantitation Results

LLOQ (0.05 ng/mL)									
LLOQ (1)	LLOQ (2)	LLOQ (3)	LLOQ (4)	LLOQ (5)	LLOQ (6)	Mean	STD	CV%	Accuracy%
0.053	0.047	0.056	0.051	0.063	0.055	**0.054**	**0.0054**	**10.00**	**108.00**

The lower limit of quantitation results show that, the analytical method of Colchicine can be measure the concentrations those not less than LLOQ accurately and precisely. [13]

3.1.3 Calibration Curve and linearity

The calibrator's concentrations covered the range from lower limit of quantitation (0.05 ng/mL) to the highest expected concentration of Colchicine in the study samples (4.00 ng/mL) as shown in figures (2, 3). The calibrators were prepared as proposed extraction procedure; the linearity was evaluated by calculating the linear regression (coefficient of determination, R^2) and by evaluating the back calculated concentrations of the calibration standards. Coefficient of determination's mean obtained from all calibration curves is 0.9985 which indicates good and accepted linearity. Calibration curve and linearity results are shown in tables 3.3 and 3.4.

3.3: Calibration Curve Linearity(1) Results

Curve no.	Slope	Intercept	R^2
1	1.84000	0.10600	0.9996
2	2.01000	0.05880	0.9986
3	1.88000	0.06330	0.9990
4	2.00000	0.03730	0.9990
5	1.68000	0.03170	0.9992
6	2.01000	0.03220	0.9998
7	2.03000	0.01260	0.9990
8	1.90000	0.02030	0.9964
9	2.02000	0.04010	0.9994

Curve no.	Slope	Intercept	R^2
10	0.92600	0.03020	0.9950
Mean	**1.82960**	**0.04325**	**0.9985**

(1) 1/X weighted factor was used

3.4: **Back Calculated Concentrations** of each Non-Zero Calibration Standard Results

Curve no.	Calibration Standard							
	Cal 01	Cal 02	Cal 03	Cal 04	Cal 05	Cal 06	Cal 07	Cal 08
	Concentrations (ng/mL)							
	0.050	**0.100**	**0.200**	**0.400**	**0.800**	**1.500**	**2.500**	**4.000**
1	0.050	0.092	0.211	0.425	0.778	1.502	2.496	3.996
2	0.042	0.115	0.185	0.408	0.853	1.497	2.543	3.907
3	0.051	0.123(1)	0.188	0.412	0.841	1.445	2.446	4.066
4	0.050	0.110	0.174	0.427	0.778	1.481	2.491	4.039
5	0.045	0.105	0.192	0.430	0.839	1.472	2.456	4.011
6	0.052	0.093	0.202	0.406	0.809	1.516	2.476	3.997
7	0.052	0.102	0.198	0.387	0.814	1.404	2.512	4.081
8	0.049	0.093	0.212	0.382	0.824	1.588	2.631	3.771
9	0.048	0.101	0.203	0.419	0.778	1.471	2.564	3.966
10	0.058	0.086	0.184	0.415	0.824	1.575	2.254	4.155
N	**10**	**9**	**10**	**10**	**10**	**10**	**10**	**10**
Mean	**0.050**	**0.100**	**0.195**	**0.411**	**0.814**	**1.495**	**2.487**	**3.999**
SD	**0.0043**	**0.0094**	**0.0124**	**0.0161**	**0.0279**	**0.0554**	**0.0986**	**0.1048**
CV%	**8.60**	**9.40**	**6.36**	**3.92**	**3.43**	**3.71**	**3.96**	**2.62**

(1) Out of acceptance range, not included in calculation.

Back calculated concentration's CV% obtained from all calibration curves indicates precise calibrators. [13]

3.1.4 Accuracy

Six quality control samples of each concentration level (LLOQ, low, med and high levels) were analyzed against a calibration curve containing standards in the quantification range. The results for within-run accuracyare given in table 3.5.

Three runs, each one consists of six quality control samples of each concentration level (LLOQ, low, med and high levels) were analyzed against a calibration curve contain standards in the quantification range. The results for between run accuracy are given in table 3.6.

The within run accuracy results indicates that, the Colchicine in human plasma can be measured with adequate accuracy at all concentration levels.

Table 3.5: Within Run Accuracy Results

Replicate		Found Concentration (ng/mL)

	QC_{LLOQ} (0.05 ng/mL)	QC_{Low} (0.15 ng/mL)	QC_{Med1} (0.60 ng/mL)	QC_{Med2} (1.60 ng/mL)	QC_{High} (3.20 ng/mL)
1	0.053	0.147	0.631	1.530	3.462
2	0.047	0.134	0.602	1.653	3.377
3	0.056	0.128	0.647	1.559	3.466
4	0.051	0.124	0.647	1.682	3.450
5	0.063	0.138	0.586	1.704	3.365
6	0.055	0.134	0.643	1.721	3.363
Mean	**0.054**	**0.134**	**0.626**	**1.642**	**3.414**
Accuracy%	**108.00**	**89.33**	**104.33**	**102.63**	**106.69**

Table 3.6: Between Run Accuracy Results

Replicate	**Found Concentration (ng/mL)**			
	QC_{LLOQ} (0.05 ng/mL)	QC_{Low} (0.15 ng/mL)	QC_{Med} (1.60 ng/mL)	QC_{High} (3.20 ng/mL)
1st Day	0.049	0.148	1.537	2.622
	0.044	0.134	1.429	2.818
	0.040	0.130	1.459	2.668
	0.047	0.131	1.664	2.626
	0.032	0.126	1.513	2.807
	0.039	0.145	1.521	2.814
2nd Day	0.058	0.151	1.595	3.247
	0.047	0.169	1.560	3.112
	0.044	0.157	1.607	3.059
	0.054	0.148	1.570	3.329
	0.048	0.160	1.531	3.122
	0.049	0.137	1.618	3.378
3rd Day	0.046	0.137	1.573	3.058
	0.052	0.147	1.540	3.049
	0.058	0.154	1.502	3.168
	0.046	0.141	1.292	3.178
	0.048	0.146	1.507	3.115
	0.051	0.139	1.228	2.996
Mean	**0.047**	**0.144**	**1.514**	**3.009**
Accuracy%	**94.00**	**96.00**	**94.63**	**94.03**

The between runs accuracy results indicate that, the analytical method of Colchicine is accurate. [13]

3.1.5Precision

Six quality control samples of each concentration level (LLOQ, low, med and high levels) were analyzed against a calibration curve containing standards in the quantification range, the results for the within-run precision are shown in table 3.7.

Table 3.7: Within Run Precision Results

Replicate		Found Concentration (ng/mL)			
	QC_{LLOQ} (0.05 ng/mL)	**QC_{Low} (0.15 ng/mL)**	**QC_{Med1} (0.60 ng/mL)**	**QC_{Med2} (1.60 ng/mL)**	**QC_{High} (3.20 ng/mL)**
1	0.053	0.147	0.631	1.530	3.462
2	0.047	0.134	0.602	1.653	3.377
3	0.056	0.128	0.647	1.559	3.466
4	0.051	0.124	0.647	1.682	3.450
5	0.063	0.138	0.586	1.704	3.365
6	0.055	0.134	0.643	1.721	3.363
Mean	**0.054**	**0.134**	**0.626**	**1.642**	**3.414**
SD	**0.0054**	**0.0080**	**0.0260**	**0.0790**	**0.0503**
CV%	**10.00**	**5.97**	**4.15**	**4.81**	**1.47**

The within run precision results indicates that, the Colchicine in human plasma can be measured with adequate precision at all concentration levels.Three runs, each one consist of six quality control samples of each concentration level (LLOQ, low, med and high levels) were analyzed against a calibration curve contain standards in the quantification range. The results for between run precision are shown in table 3.8.

Table 3.8: Between Run Precision of Colchicine

Replicate	Found Concentration (ng/mL)			
	QC_{LLOQ} (0.05 ng/mL)	**QC_{Low} (0.15 ng/mL)**	**QC_{Med} (1.60 ng/mL)**	**QC_{High} (3.20 ng/mL)**
1st Day	0.049	0.148	1.537	2.622
	0.044	0.134	1.429	2.818
	0.040	0.130	1.459	2.668
	0.047	0.131	1.664	2.626
	0.032	0.126	1.513	2.807
	0.039	0.145	1.521	2.814
2nd Day	0.058	0.151	1.595	3.247
	0.047	0.169	1.560	3.112
	0.044	0.157	1.607	3.059

Replicate	Found Concentration (ng/mL)			
	QC_{LLOQ} (0.05 ng/mL)	QC_{Low} (0.15 ng/mL)	QC_{Med} (1.60 ng/mL)	QC_{High} (3.20 ng/mL)
	0.054	0.148	1.570	3.329
	0.048	0.160	1.531	3.122
	0.049	0.137	1.618	3.378
3rd Day	0.046	0.137	1.573	3.058
	0.052	0.147	1.540	3.049
	0.058	0.154	1.502	3.168
	0.046	0.141	1.292	3.178
	0.048	0.146	1.507	3.115
	0.051	0.139	1.228	2.996
Mean	**0.047**	**0.144**	**1.514**	**3.009**
SD	**0.0064**	**0.0113**	**0.1086**	**0.2324**
CV%	**13.62**	**7.85**	**7.17**	**7.72**

The between runs precision results indicate that, the analytical method of Colchicine is precise. [13]

3.1.6 Stability

3.1.6.1 Freeze and Thaw Stability

Colchicine stability was determined for four freeze and thaw cycles. Three aliquots at each of the QC_{Low} and QC_{High} concentrations were stored at -20℃ for at least 12 hours and thawed unassisted at room temperature. When completely thawed, the samples refrozen for at least 12 hours under the same conditions.
The samples were analyzed after the 4th cycle, and the results are shown in table 3.9.

Table 3.9: Freeze - thaw cycle stabilityof Colchicine

Stability Condition	Colchicine (QC_{Low}) (0.15 ng/mL)		Colchicine (QC_{High}) (3.20 ng/mL)	
	-20 °C		-20 °C	
Replicate	**Fresh**	**4th cycle**	**Fresh**	**4th cycle**
1	0.146	0.177	3.417	3.386
2	0.154	0.169	3.418	3.203
3	0.162	0.152	3.406	3.317
Mean	**0.154**	**0.166**	**3.414**	**3.302**
Stability %	**110.67**		**103.19**	

The stability results indicate that, the Colchicine is stable for four freeze and thaw cycles at-20 °C.

3.1.6.2 Short Term Stability

Three aliquots of each of the QC_{Low} and QC_{High} concentrations thawed unassisted at room temperature and kept for 18:44 hours: minutes, based on the expected duration that samples will be maintained at room temperature in any intended study, the results are obtained in table 3.10

Table 3.10: Short Term Stability of Colchicine

Stability Condition	**Colchicine (QC_{Low}) (0.15 ng/mL)**		**Colchicine (QC_{High}) (3.20 ng/mL)**	
	R.T		**R.T**	
Replicate	**Fresh**	**18:44 (hr:min)**	**Fresh**	**18:44 (hr:min)**
1	0.146	0.157	3.417	3.152
2	0.154	0.195	3.418	3.234
3	0.162	0.157	3.406	2.926
Mean	**0.154**	**0.170**	**3.414**	**3.104**
Stability %	**113.33**		**97.00**	

It's concluded that, the Colchicine is stable in plasma for 18:44 hours: minutes at room temperature before processing.

3.1.6.3 Long Term Stability

The storage time in the long-term stability evaluated to cover the part of the period between the date of first sample collection and the date of last sample analysis. Long-term stability determined by storing at least three aliquots of each of the QC_{Low} and QC_{High} concentrations at -20 ºC for 86 days then analyzed, the results are shown in table 3.11.

Table 3.11: Long Term Stability of Colchicine

Stability Condition	**Colchicine (QC_{Low}) (0.15 ng/mL)**		**Colchicine (QC_{High}) (3.20 ng/mL)**	
	-20 °C		**-20 °C**	
Replicate	**Fresh**	**86 Days**	**Fresh**	**86 Days**
1	0.147	0.132	3.462	3.330
2	0.134	0.137	3.377	3.418
3	0.128	0.125	3.466	3.414
Mean	**0.136**	**0.131**	**3.435**	**3.387**
Stability %	**87.33**		**105.84**	

It's concluded that Colchicine plasma samples are stable for 86 days when stored frozen at -20ºC.

3.1.7 Recovery

The recovery of Colchicine and Colchicine- D_3 was determined on the three concentration levels of QCs (QC_{Low}, QC_{Med} and QC_{High}) by comparing extracted QC samples with pure solution samples. CV% of Recovery of Colchicine and internal standard (Colchicine- D_3) in (low, Med, High) should be less than 20.00%, the results are illustrated in tables (3.12 and 3.13)

Table 3.12: Absolute Recovery of Colchicine

Replicate	QC_{Low} (Area)		QC_{Med} (Area)		QC_{High} (Area)	
	Plasma	**Solution**	**Plasma**	**Solution**	**Plasma**	**Solution**
1	5202	7079	43573	76085	87301	150124
2	4552	7216	42439	75801	53105	169667
3	3755	7584	43036	71007	80802	173012
Mean	**4503**	**7293**	**43016**	**74298**	**73736**	**164268**
Recovery %	**61.74**		**57.90**		**44.89**	
Mean	**54.84**					
SD	**8.831**					
CV%	**16.10**					

Table 3.13: Absolute Recovery of Colchicine- D_3

Replicate	IS (Area) in QC_{Low}		IS (Area) in QC_{Med}		IS (Area) in QC_{High}	
	Plasma	**Solution**	**Plasma**	**Solution**	**Plasma**	**Solution**
1	13120	18396	12782	20383	12579	19858
2	12745	18855	12187	19767	7205	22897
3	10065	20313	11787	19617	11795	22586
Mean	**11977**	**19188**	**12252**	**19922**	**10526**	**21780**
Recovery %	**62.42**		**61.50**		**48.33**	
Mean	**57.42**					
SD	**7.883**					
CV%	**13.73**					

4. Conclusion

The currentLC/MS method which was developed for determination of Colchicine in human plasma is simple, selective, sensitive and stable. The method employing simple liquid–liquid extraction for sample preparation enables rapid and very simple quantification of Colchicine in human plasma in the range (0.05–0.40) ng mL−1. fully validation process in accordance with EMEA guidelines were successfully established. The accuracy, precision, and sensitivity of the method are within the accepted range, the method has very short run time (about 0.80 min). In addition, the current LC/MS chromatographic methodcould be used in clinical studies for analysis of Colchicine in healthy volunteers and pharmacokinetic application in biological fluids.

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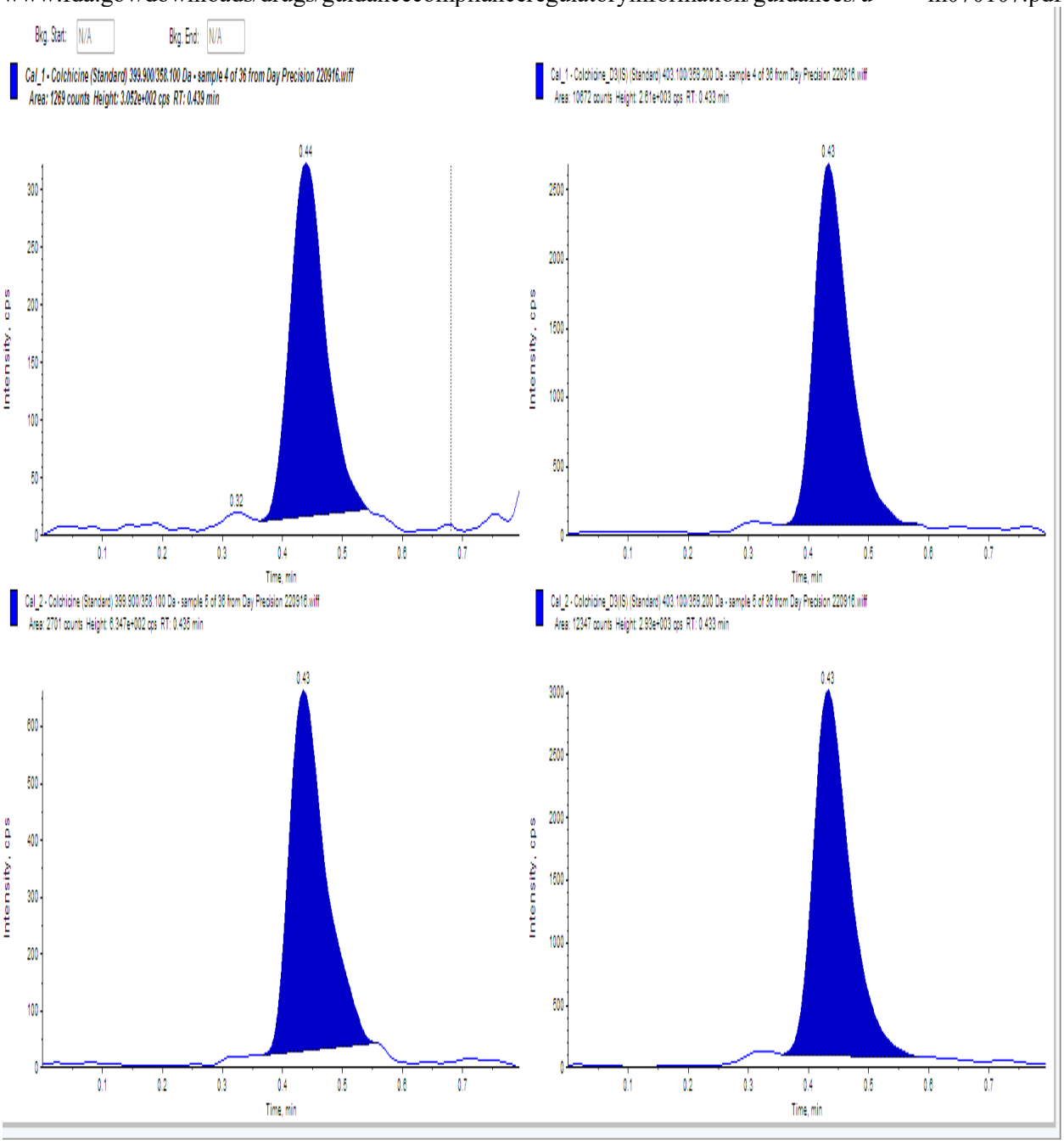

Fig. 2: (A) LLOQ (1st Calibrator 0.05 ng/mL) (B) 2nd Calibrator 0.10 ng/mL

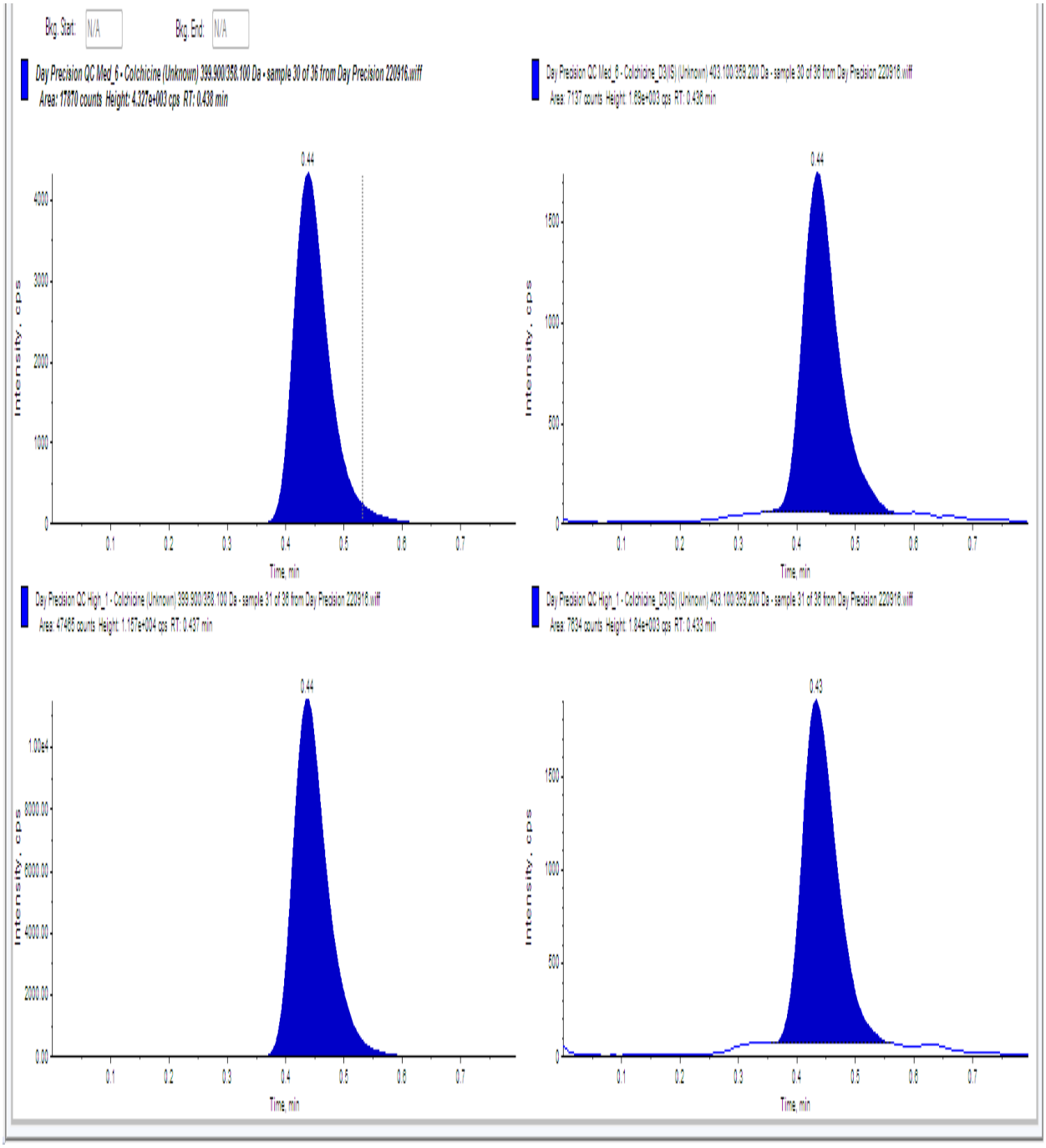

Fig 3: (A) QC_{Med} 1.60 ng/mL (B) QC_{High} 3.20 ng/mL

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