Research Article

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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF GRISEOFULVIN IN K3EDTA HUMAN PLASMA BY LC-MS/MS

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ABSTRACT

A sensitive liquid chromatography- mass spectroscopy (LC-MS/MS) method is developed and validated for rapid determination of Griseofulvin In K_3EDTA Human Plasma. Griseofulvin D_3 was used as the internal standard (I.S). Withdrawn blank, calibration curve standards, quality control samples and subject samples from the deep freezer and allowed them to thaw. Vortexed the thawed samples to ensure complete mixing of contents. Transferred 0.25ml of human plasma sample in to a Ria vial; add 25µl of Griseofulvin-d3 (approximately 10.000µg/ml). In the blank samples and predose samples ((0.00hr), added 25µl of 80% acetonitrile in water solution .Vortexed the samples to ensure mixing of contents completely. Added 2.500ml of extraction solvent (Dichloromethane: Diethyl ether 20:80), place on a shaker for 20 minutes and centrifuge for 10 minutes at 4000rpm at 20°c and transfer the supernatant (organic layer) in to another ria vial. Evaporated this layer under a

stream of nitrogen gas at 50°c. Reconstituted the residue with 0.250 ml of mobile phase and vortex. Loaded the sample into auto-injector vials. Injected 15µL onto LC-MS/MS system.

Key Words: Griseofulvin, K_3EDTA, Human plasma, Ria vial, Nitrogen gas.

INTRODUCTION:

The bioanalytical methods used to determine the drug and/or its metabolites in the plasma, serum, blood or urine or any other suitable matrix must be well characterized, standardized, fully validated and documented to yield reliable results that can be satisfactorily interpreted.

Drug profile:

Griseofulvin is a antifungal-antibiotic. Its chemical name is (2S,6^{1}R)-7-Chloro-2^1,4,6-trimethoxy-6^1-methyl-3H,4^{1}H-Spir0(1-benzofuran-2,11-cyclohex(2)ene)-3,4-dione. And its molecular formula is $C_{17}H_{17}ClO_6$ and its molecular weight is 352.77 gm/mol. Griseofulvin is fungistatic with *in vitro* activity against various species of Microsporum, Epidermophyton and Trichophyton. It has no effect on bacteria or other genera of fungi.The drug binds to tubulin, interfering with microtubule function, thus inhibiting mitosis.

Experimental Method

Reagents & Requirements

Griseofulvin, Griseofulvin D_3,Formic acid, Acetonitrile, Dichloromethane, Methanol, Diethyl ether, HPLC grade Water, Micro balance, Micro pipette, Glass bottles, Volumetric flask, Vortexer, Deep freezer (-20±5°C), Deep freezer(-70±15°c), Nitrogen evaporator, Refrigerator, SPE Positive pressure, Pipette tips, Surgical gloves, Combitips, Cartridge, RIA vial.

Preparation of Solutions

Preparation of Griseofulvin standard stock solution:

Weight and transfer Griseofulvin working standard equivalent to 5.000 mg of

Griseofulvin into a 5.000 mL volumetric flask and dissolve in methanol. Make up the volume with the same. Prepare the stock for CCs and QCs spiking separately. Calculate the concentration of resulting solutions by considering the purity of Griseofulvin. Label and store the solutions at 2-8ºC.

Preparation of Internal standard stock solution:

Weight and transfer Griseofulvin-d3 working standard equivalent to 2.000 mg of Griseofulvin-d3 into a 2.000 mL volumetric flask and dissolve in acetonitrile. Make up the volume with the same. Calculate the concentration of resulting solution by considering the purity of Griseofulvin-d3. Label and store the solution at 2-8ºC. Then fill the ´Stock Weighing and solution Preparatioǹ form. Dilute the stock solution with 80% Acetonitrile in water solution as and when required to get a concentration of about 10.000 µ / ml.

Preparation of Reference Standard Solution: Prepare a mixture of 1.000 µg/mL concentration of Griseofulvin and 1.000 µg/mL concentration of ISTD (Griseofulvin-d3) in mobile phase.

Preparation of 0.05% Formic acid Buffer (pH 5.5):

Transfer 585 µL of Formic acid in 1000.000 mL of water and sonicate. Label and store the solution at ambient temperature.

Preparation of Mobile phase:

To 300.000 mL of above 0.05% Formic acid buffer, add 700.000 mL of Acetonitrile mix well and sonicate. Label and store the solution at ambient temperature.

Perparation of 10% Acetonitrile in water solution: To 100.000 mL of acetonitrile, add 900.000 mL of water and sonicate. Label and store the solution at ambient temperature.

Preperation of 80 % Acetonitrile in water solution:

To 80.000 mL of Acetonitrile, add 20.000 mL of water and sonicate. Label and store the solution at ambient temperature.

Perparation of 90% Acetonitrile in water solutionL:

To 900.000 mL of acetonitrile, add 100.000 ml of water and sonicate. Label and store the solution at ambient temperature.

Preparation of Extraction solvent:

To 200.000 mL of Dichloromethane, add 800.000 mL of Diethyl ether and sonicate. Label and store the solution at ambient temperature.

Preparation of Calibration Curve (CC) Standards:

Preparation of stock dilutions of standard Griseofulvin solution:

Prepared the following stock dilutions of Griseofulvin ranging from 0.400 µg/mL to 60.056 µg/mL with 80 % acetonitrile in water solution using dilutions of main stock solution prepared for calibration curve standards, shown in table 1.

Calibration curve (CC) standards:

Table No: 1

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Diluent (mL)	Final volume (mL)	Final Conc. (µg/mL)
1000.928	0.120	1.880	2.000	60.056
1000.928	0.080	1.920	2.000	40.037
100.093	0.400	1.600	2.000	20.019
100.093	0.200	1.800	2.000	10.009
100.093	0.100	1.900	2.000	5.005
10.009	0.400	1.600	2.000	2.002
10.009	0.160	1.840	2.000	0.801
10.009	0.080	1.920	2.000	0.400

Spiking of plasma for calibration curve standards:

Prepared the following concentrations of Griseofulvin ranging from 20.000 ng/mL to 3002.800 ng/mL with K_3EDTA human plasma using final concentrations from above table and labeled them as CC1 to CC8 shown in table 2.

Table No: 2-Preparation of Calibration Curve Standards:

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Diluent (mL)	Final volume (mL)	Final Conc. (µg/mL)	Label
60.056	0.150	2.850	3.000	3002.800	CC8
40,037	0.150	2.850	3.000	2001.850	CC7
20.019	0.150	2.850	3.000	1000.950	CC6
10.009	0.150	2.850	3.000	500.450	CC5
5.005	0.150	2.850	3.000	250.250	CC4
2.002	0.150	2.850	3.000	100.100	CC3
0.801	0.150	2.850	3.000	40.050	CC2
0.400	0.150	2.850	3.000	20.000	CC1

Preparation of Quality Control (QC) samples:

Preparation of stock dilutions of standard Griseofulvin solution:

Prepare the stock dilutions of Griseofulvin ranging from 0.401µg/ml to 50.106µg/ml with 80% acetonitrile in water solution using dilutions of main stock solution prepared for quality control samples shown in table 3.

Table No: 3-Preparation of Quality control (QC) samples:

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Diluent (mL)	Final volume (mL)	Final Conc. (µg/mL)
1002.118	0.250	4.750	5.000	50.106
1002.118	0.150	4.850	5.000	30.064
100.212	0.300	4.700	5.000	6.013
10.021	0.600	4.400	5.000	1.203
10.021	0.200	40800	5.000	0.401

Spiking of plasma for quality control samples:

Prepare the following concentrations of Griseofulvin ranging from 20.050 ng/mL to 2505.300 ng/mL with K_3EDTA human plasma using final concentrations from above table and labeled them as lower limit of quantitation (LLOQ), low concentration (LQC), Geometric mean concentration (GMQC), medium concentration (MQC) and high concentration (HQC) quality control samples respectively, shown in table 4.

Table No: 4 Preparation of Quality Control (QC) Samples:

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Plasma (mL)	Final volume (mL)	Final Conc. (ng/mL)	Label
50.106	1.250	23.750	25.000	2505.300	HQC
30.064	1.250	23.750	25.000	1503.200	MQC
6.013	1.250	23.750	25.000	300.650	GMQC
1.203	1.250	23.750	25.000	60.150	LQC
0.401	0.250	4.750	5.000	20.050	LLOQ

Spiking with Haemolytic plasma for quality control samples:

Prepared the following concentrations of Griseofulvin of 60.150 ng/mL and 2505.300 ng/mL with Haemolytic K3EDTA plasma using final concentrations from the above table and labelled them as Haemolytic LQC and Haemolytic HQC respectively, shown in table 5.

Table No: 5- Preparation of Quality Control (QC) Samples:

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Haemolytic Plasma (mL)	Final volume (mL)	Final Conc. (ng/mL)	Label
50.106	1.250	23.750	25.000	2505.300	Haemolytic HQC
1.203	1.250	23.750	25.000	60.150	Haemolytic LQC

Spiking of Lipemic plasma for quality control samples:

Prepare the following concentrations of Griseofulvin 60.150 ng/mL and 2505.300 ng/mL and Lipemic K3EDTA plasma using final concentrations from the above table and labelled them as Lipemic LQC and Lipemic HQC respectively. Shown in table 6.

Table No: 6 Preparation of Quality Control (QC) Samples:

Stock Conc. (µg/mL)	Volume of Stock (mL)	Volume of Lipemic Plasma (mL)	Final volume (mL)	Final Conc. (ng/mL)	Label
50.106	1.250	23.750	25.000	2505.300	Lipemic HQC
1.203	1.250	23.750	25.000	60.150	Lipemic LQC

DEVELOPED METHOD:

Biological matrix	Human plasma
Anticoagulant	K3 EDTA
volume of injection	15µL
Analyte	Griseofulvin
Internal standard	Griseofulvin $-d_3$
Analytical technique	Liquid chromatography
Detection mode	Mass spectrometer
Extraction procedure	Liquid phase extraction
Quantitation method	Peak area
Weighing method	$1/X^2$

Chromatographic conditions:

Chromatographic mode	LC/MS/MS-API-2000
Mobile phase	Formic acid buffer: Acetonitrile(30:70)
Buffer	0.5% formic acid in HPLC water
Column	C18
Isocratic/gradient mode	Isocratic mode
Mobile phase flow rate	1.000 ml/min
Auto sample temperature	10°c
Syringe speed	5µl/sec
Rinsing volume	600µl
Column temperature	40°c
Injection volume	15µl

RETENTION TIMES:

Griseofulvin : 0.80 to 1.60

ISTD : 0.80 to 1.60

Table 7- DETECTION PARAMETERS

Drug name	Griseofulvin	Griseofulvin- d_3
Parent mass	353.2*	356.1*
Product mass	215.0*	168.1 *

Table 8- MULTIPLE REACTIONS MONITORING (MRM)

Curtain Gas (CUR)	20 PSI
Collision Gas (CAD)	4 PSI
Nebulizer Current	2.0
Temperature (TEM)	$500^{O}C$
GAS-1	40 PSI
GAS-2	50 PSI
Collision Energy (CE)	30 V
Entrance Potential (EP)	10 V
Focusing Potential (FP)	300 V

PARAMETERS	**Griseofulvin**	**ISTD (Griseofulvin-D_3)**
Declustering potential (DP)	26 V	30 V
Collision Cell Exit Potential (CXP)	6 V	5 V

Resolution Q1 : Unit

Q3 : Unit

DATA COLLECTION AND COMPUTER SYSTEM:

All integrations were performed by Applied Bio systems Analyst® soft ware version 1.4.2. The slopes, intercepts, and correlation coefficients were determined by least squares linear regression analysis using the ratios of drug/internal standard peak areas of Calibration curve standards.

All the QC samples were also calculated by Applied Bio-systems Analyst® Software version 1.4.2, Canada.

The concentrations of the unknown sample have to be calculated from the equation using regression analysis of spiked plasma calibration curve standard with $1/x^2$ as the weighting factor.

Y= mx+c

Where,

y = Ratio of Griseofulvin peak area and Griseofulvin-d_3

x = concentration of Griseofulvin

m = Slope of calibration curve

c = y-axis intercept value

SAMPLE PROCESSING:

Withdraw blank, calibration curve standards, quality control samples and subject samples from the deep freezer and allow them to thaw. Vortex the thawed samples to ensure complete mixing of contents. Transfer 0.25ml of human plasma sample in to a Ria vial; add 25µl of Griseofulvin-d3 (approximately 10.000µg/ml). In the blank samples and predose samples ((0.00hr), add 25µl of 80% acetonitrile in water solution .Vortex the samples to ensure mixing of contents completely.

Add 2.500ml of extraction solvent (Dichloromethane: 20:80), place on a shaker for 20 minutes and centrifuge for 10 minutes at 4000rpm at 20°c and transfer the supernatant (organic layer) in to another ria vial. Evaporate this layer under a stream of nitrogen Diethyl ether gas at 50°c. Reconstitute the residue with 0.250 ml of mobile phase and vortex. Load the sample into auto-injector vials. Inject 15µL onto LC-MS/MS system.

VALIDATION AND METHODS:

System suitability 1:

The results of system suitability have been tabulated in tables. The results were within the acceptance criteria.

Table No. 9

S.No	RT(in minutes)		Peak response (area)		
	Analyte	Internal Standard	Analyte	Internal standard	Analyte area/ISTD area
1	1.24	1.24	145785	191131	0.76
2	1.25	1.24	146926	200956	0.73
3	1.25	1.24	149663	199737	0.75
4	1.24	1.24	146820	201897	0.73
5	1.25	1.24	146926	204397	0.72
6	1.25	1.24	150701	203280	0.74
Mean	1.25	1.24			0.74
SD(±)	0.0052	0.0000			0.0160
CV %	0.42	0.00			2.17

Table No. 10-System Suitability -2

S.No	RT(in minutes)		Peak response (area)		
	Analyte	Internal Standard	Analyte	Internal standard	Analyte area/ISTD area
1	1.25	1.24	206811	223587	0.92
2	1.25	1.24	217050	237806	0.91
3	1.25	1.24	204720	233687	0.88
4	1.25	1.24	213355	238780	0.89
5	1.25	1.24	209140	231644	0.90
6	1.25	1.24	210885	233671	0.90
Mean	1.25	1.24			0.90
SD(±)	0.0000	0.0000			0.0167
CV %	0.00	0.00			1.85

PRECISION AND ACCURACY:

The % accuracy observed for inter-batch QC samples was 103.02 %, 98.39%, 99.05 % for LQC, GMQC, MQC and HQC respectively.

The percentage for intra-batch QC samples was ranged from 101.3% to 104.08%, 95.20% to 100.31%, 95.87% to 101.26% and 87.34% to 92.97% for LQC, GMQC, MQC, and HQC respectively. The CV% observed for the inter-batch QC sample was 4.32%, 3.67%, 3.89% and 3.47% for LQCC, GMQC, MQC and HQC respectively. The CV% for the inter-batch QC samples ranged from 3.19% to 5.32%,

1.83% to 3.56%, 2.31% to 4.69% and 2.27% to 2.42% for LQC, GMQC, MQC and HQC respectively.

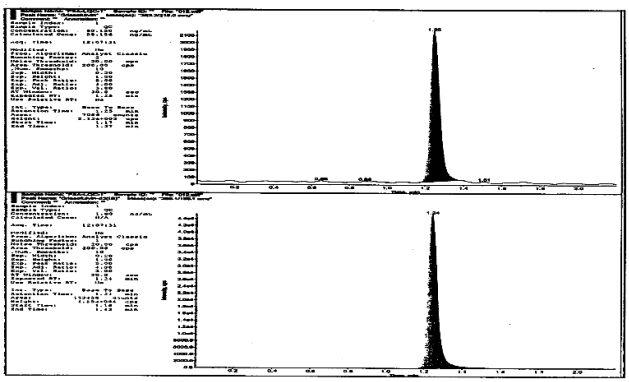
In Haemolytic Plasma:

The % accuracy observed for QC samples spiked using Haemolytic plasma was 97.71% and 94.60% for Haemolytic LQC and Haemolytic HQC respectively. The CV% observed for QC samples spiked with Haemolytic plasma was 5.65% and 2.82% for Haemolytic LQC and Lipemic HQC respectively.

In Lipemic Plasma:

The % accuracy observed for QC samples spiked using Lipemic plasma was 106.74% and 93.59% for Lipemic LQC and Lipemic HQC respectively. The CV% observed for QC samples spiked with Lipemic plasma was 5.65% and 2.82% for Haemolytic LQC and Lipemic HQC respectively.

Chromatogram of LQC



Chromatogram of GMQC

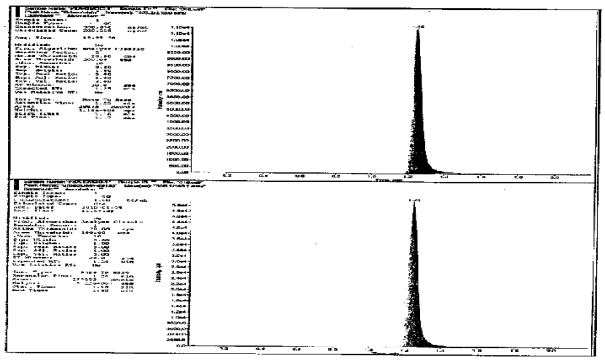


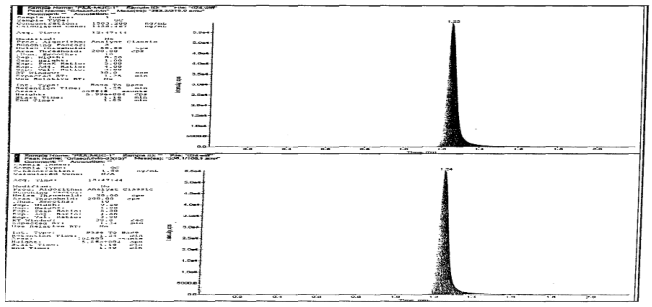
Table No .11-INTER-BATCH PRECISION AND ACCURACY (GLOBAL STATISTICS THREE P&A BATCHES:

Batch	ID	LQC (ng/mL)	GMQC (ng/mL)	MQC (ng/mL)	HQC (ng/mL)
I	**P&A-1**	58.156	288.558	1425.467	2125.412
	P&A-2	63.620	303.866	1385.587	2150.730
	P&A-3	59.830	276.514	1495.115	2257.582
	P&A-4	64.950	286.733	1441.729	2163.970
	P&A-5	58.631	275.785	1358.792	2223.805
	P&A-6	60.689	285.895	1540.023	2207.454
II	**P&A-7**	57.172	293.159	1524.658	2298.924
	P&A-8	64.534	306.047	1498.748	2403.689
	P&A-9	61.528	284.401	1552.731	2278.622
	P&A-10	65.960	296.966	1564.003	2380.020
	P&A-11	64.585	306.357	1467.885	2347.715
	P&A-12	60.169	310.941	1524.980	2266.287
III	**P&A-13**	59.912	300.895	1520.996	2313.134
	P&A-14	62.438	304.063	1533.051	2245.485
	P&A-15	62.540	302.517	1509.817	2306.171
	P&A-16	64.012	308.358	1504.368	2348.445
	P&A-17	61.170	291.684	1519.599	2296.799
	P&A-18	65.539	301.925	1434.108	2197.458
Mean		61.969	295.815	1488.981	2267.317
SD(±)		2.6749	10.8622	57.8727	78.6988
CV %		4.32	3.67	3.89	3.47
% Accuracy		103.02	98.39	99.05	90.50
Actual Conc. ng/Ml		**60.150**	**300.650**	**1503.200**	**2505.300**

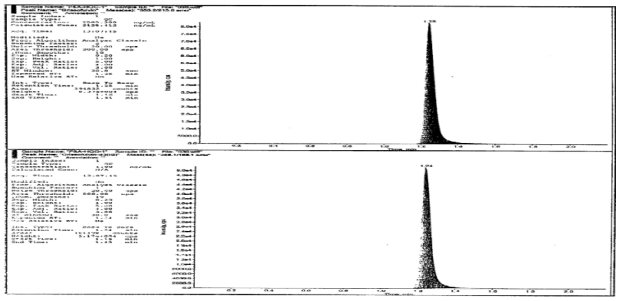
Results and Discussion:

Calibration curve range	20.00 to 3002.800 ng/ml.
Intra Batch Accuracy	101.38%% to 92.97%.
Intra Batch Precision	3.19% to 2.42%.
Inter Batch Accuracy	103.02% to 90.50%.
Inter Batch Precision	4.32% to 3.47%.
Bench Top Stability after 14 hrs %stability	LQC-103.33%, HQC -94.57%
Dry Extract Stability after 20 hrs ,%stability	LQC-103.78% ,HQC-96.64% LQC -0.35%, HQC 3.60%.

Chromatogram of MQC:



Chromatogram of HQC:



SUMMARY AND CONCLUSION:

In the present study, the method validation and system suitability were performed for the Griseofulvin in K_3EDTA human plasma by LC-MS/MS and results obtained were within the acceptance criteria (CV%≤3 for area ratio & CV%≤2 for RT). Based on the results of validation, it can be concluded that the present method is suitable for the estimation of Griseofulvin in K_3EDTA human plasma over concentration range of 20.000 ng/ml to 3002.800 ng/mL. The precision and accuracy are very much within the prescribed limits in this concentration range. The drug was found to be very stable to the effect of three freeze-thaw cycles, up to 14.00 hours delay on the Bench-top, up to 20.00 hrs of Wet extract stability. This method is found to be rugged with changes in column and analyst.

Limit ranges as per IP 85-115%.

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