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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF NITROFURANTOIN IN HUMAN PLASMA BY LC -MS / MS

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ABSTRACT: A simple, rapid and sensitive method using an isocratic Liquid chromatography coupled with Tandem mass spectrometry was developed and validated for the assay of nitrofurantoin in the Human Plasma. The Mass transition of nitrofurantoin and losartan (Internal standard) were M/z 237.1/151.9 and M/Z 421.300/ 179 in ESI Negative ionization. Linearity was observed between the nitrofurantoin concentration and the peak area ratio from 10.451 to 1033.897 ng/mL with R2 value of 0.99. Plasma samples containing nitrofurantoin were extracted with Acetonitrile: 5mM Ammonium Acetate (pH 3.8) (80:20). The observed recovery of nitrofurantoin was 90%. The intra-day and inter-day accuracy was performed. Stability parameter was performed. The method will be used in the determination of the pharmacokinetic parameters of nitrofurantoin.

INTRODUCTION: Nitrofurantoin is an antibiotic that has fights bacteria in the body. Chemically it is (*E*)-1-[(5nitro2furyl)methylideneamino] imidazolid ine-2,4-dione [1 - 4]. It has an empirical formula of $C_8H_6N_4O_5$ and a molecular weight of 238.16 g/mol. It works as an antibiotic by damaging bacterial DNA, since its reduced form is highly reactive. It is used in the treatment of UTI's caused by susceptible bacteria nitrofurantoin is readily absorbed after oral dosing from the gastrointestinal tract with peak plasma levels occurring in 4 - 8 hrs and half life is 20 - 60 minutes. Literature survey reveals that very few methods were developed and validation for the quantification of nitrofurantoin in pharmaceutical and biological fluids [5 - 13].

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The aim and objective of proposed method is to develop the simplest, sensitive, high recovery and selective method with proper internal standard usage. The method was validated as per ICH guidelines [14].

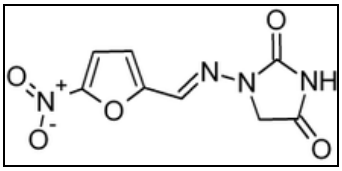


FIG. 1: STRUCTURE OF NITROFURANTOIN

MATERIALS AND METHODS:

Instrumentation: A thermo LC system equipped was used to inject 25µl of the samples on a Hypurity advance Kromosil-C-8; 4.6x50mm which was kept at ambient temperature of 25 °C. The Electron Spray Ionization source was heated at 4500 °C nebulizer operating in negative ion mode. A mixture of buffer and acetonitrile in the ratio of 20:80 % v/v was degassed ultrasonically for 5 min. The flow rate of mobile phase was 0.8 ml/min (split ratio-0.5ml).

Preparation of Standard Stock and Plasma Samples: The nitrofurantoin standard stock solution of 1033896.6 ng/ml was prepared by dissolving required quantity in acetonitrile and dimethyl sulphoxide. This was further diluted with acetonitrile and water to get a concentration of 51694.8 ng/ml. The spiked calibration curve standards (CC) and quality control samples (QC) were prepared by using standard stock and intermediate stock solution in 80% acetonitrile in water. The internal standard (IS) stock solution was prepared by dissolving 10 mg in methanol. This was further diluted with methanol to get a concentration of 1033896.6 ng/ml. all the solutions were stored at - 20 °C. The CC standards and QC samples were prepared by spiking human plasma with respective working solutions. CC standards were prepared at 10.4505, 26.1262, 52.2524, 104.5048, 261.2621, 466.5394, 681.0794, 878.8121 and 1033.8966 n/ml for nitrofurantoin. QC samples were prepared at 10.5163 ng/ml (LLOQ QC), 28.8119 ng/ml (LQC), 364.7070 ng/ml (MQC) and 878.8121 ng/ml (HQC) concentrations as shown in the **Table 1** and **2**. All the spiked samples were stored at -20 °C for sample analysis and validation.

TABLE 1: PREPARATION OF SPIKED CALIBRATION CURVE STANDARDS

Stock CC-ID	Stock Concentration (ng/ml)	Stock Aliquot (ml)	Plasma Added (ml)	Final Volume (ml)	Final Concentration (ng/ml)	Spiked CC-ID
SS1	51694.8300	0.2	9.8	10	1033.8966	I/I1
SS2	43940.6055	0.2	9.8	10	878.8121	H
SS3	34053.9693	0.2	9.8	10	681.0794	G
SS4	23326.9689	0.2	9.8	10	466.5394	F
SS5	13063.1026	0.2	9.8	10	261.2621	E
SS6	5225.2410	0.2	9.8	10	104.5048	D
SS7	2612.6205	0.2	9.8	10	52.2524	C
SS8	1306.3103	0.2	9.8	10	26.1262	B
SS9	522.5241	0.2	9.8	10	10.4505	A/A1

TABLE 2: PREPARATION OF SPIKED QUALITY CONTROL SAMPLES

Stock CC-ID	Stock Concentration (ng/ml)	Stock Aliquot (ml)	Plasma Added (ml)	Final Volume (ml)	Final Concentration (ng/ml)	Spiked CC-ID
QC1	43940.6055	0.2	9.8	10	878.8121	HQC
QC2	18235.3513	0.2	9.8	10	364.7070	MQC
QC3	1440.5928	0.2	9.8	10	28.8119	LQC
QC4	525.8164	0.2	9.8	10	10.5163	LLOQ

Sample Preparation: Prior to analysis, the required number of quality Control samples along with the Calibration Curve Standards was withdrawn from Ultra Low Temperature Freezer and thawed to room temperature. Thawed samples were vortexed ensuring complete mixing of contents.

500µl of samples and 50µl of Internal Standard (10000ng/ml) solution was added to all samples except blank and vortexed. add 50µl of 10% v/v Orthophosphoric acid and 2.5 ml of ethyl acetate was added to all samples and vortexed and vibramaxed at 2500 rpm for 10 minutes centrifuged at 4500rpm for 10 minutes at 4 °C in a refrigerated Centrifuge from that take 1.8 ml of supernatant samples were separated and taken in fresh ria vial were dried under nitrogen evaporator at 50 °C and 15 psi. The dried residue sample in all RIA vials was reconstituted with 0.5ml mobile phase and vortexed and injects 25µl of solution into LC-MS/MS system.

Validation Procedures: The method was validated as per US FDA guidelines. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of drug (LLOQ) and IS during the start of the method validation. The carryover test of the auto sampler was performed by injecting a sequence of injections consisting of standard (LLOQ, ULOQ), reconstituted solution, and standard blank and extracted standard equivalent to highest standard in the CC alternatively to check there is any carryover in the bank sample. The selectivity of the method was established by checking the blank K2 EDTA human plasma, K2 EDTA lipemic plasma and K2 EDTA haemolytic

plasma obtained from 8 different donors. Also spiked six samples at LLOQ concentration of nitrofurantoin and IS in plasma of one donor from above plasmas (except hemolytic and lipemic plasmas). Compare the response of analyte and IS in blanks with the mean response of injected LLOQ. The matrix effect for the intended method was assessed by using chromatographically screened human plasma concentrations equivalent to LQC and HQC prepared with six different lots. The linearity of the method was determined by using a $1/x^2$ weighted least square regression analysis of standard plots associated with a 9 point standard curve. The intra and inter day accuracy and precision were determined by analyzing 6 replicates of LQC, GMQC, MQC and HQC in a single day and between two consecutive days respectively. The precision (% CV) at each concentration level should not be more than 15% except for LLOQ QC where it should be 20%. The accuracy (%) must be within +15% of their nominal value except for LLOQ QC where it should be within +20%. The percentage mean recoveries were determined by measuring the concentrations of the extracted plasma QC samples at HQC, MQC and LQC against unextracted QC samples. Reinjection reproducibility was performed by analyzing 6 replicates of LQC and HQC samples. Stability results were assessed by measuring the area response of stability samples against freshly prepared LQC and HQC samples. Freeze thaw stability was estimated after 3 cycles of freezing and thawing of samples at -20 °C auto sampler stability was determined by keeping the samples in the auto sampler at 15 °C and analyzed after 36 hrs under a fresh calibration curve. Dry extract stability was evaluated by reconstituting and evaporating the samples kept on the bench at ambient temperature and analyzed after 24hrs.

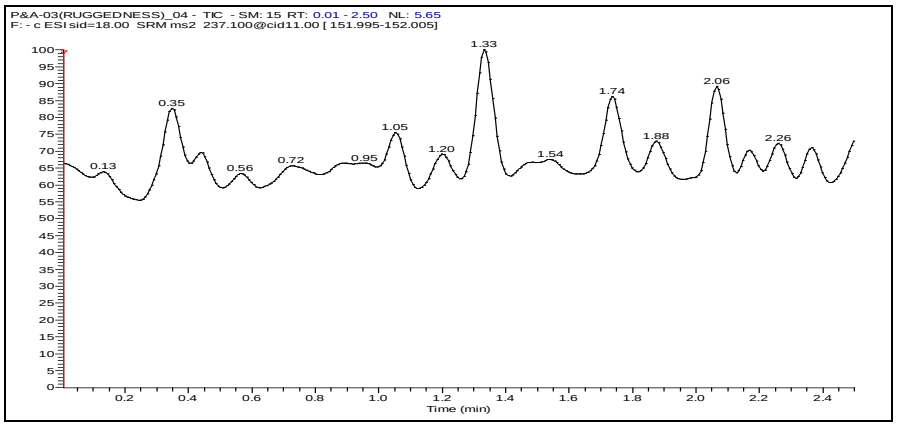
RESULTS AND DISCUSSION:

Optimization of Liquid Chromatography and Mass Spectrometry Conditions: For complete chromatographic resolution of nitrofurantoin and Internal Standard, several solvents such as acetonitrile, methanol, different buffers and mixture of solvents were tried along with different flow rates. Finally the resolution of peaks was achieved with 5mM ammonium acetate (pH 3.8) in Acetonitrile: 5mM Ammonium Acetate with pH (3.8), (80:20) using High purity advance Kromosil-C-8; 4.6x50mm (25µl). The flow rate was 0.8ml/min. Following mass spectrometric conditions, precursor ion to the parent ion transitions for nitrofurantoin and IS were at m/z 237.1→151.9 and 421.3→179.0 respectively was used for quantification purpose.

Method Validation Parameters: The selectivity of the present method was established by checking any interfering compounds that elute along with nitrofurantoin. The response of analyte and IS in blanks was compared with the mean response of injected LLOQ. Hence there were no interfering peaks formed at nitrofurantoin retention time and IS retention time in the plasma blanks. **Fig.1** shows representative chromatograms of K2 EDTA blank human plasma samples.

The specificity of the method was determined by comparing the response of analyte and IS with the mean response of injected LLOQ. There were no interfering peaks obtained at nitrofurantoin and IS retention time. The results were shown in **Table 3**.

Chromatogram of Blank Plasma and Internal Standard:



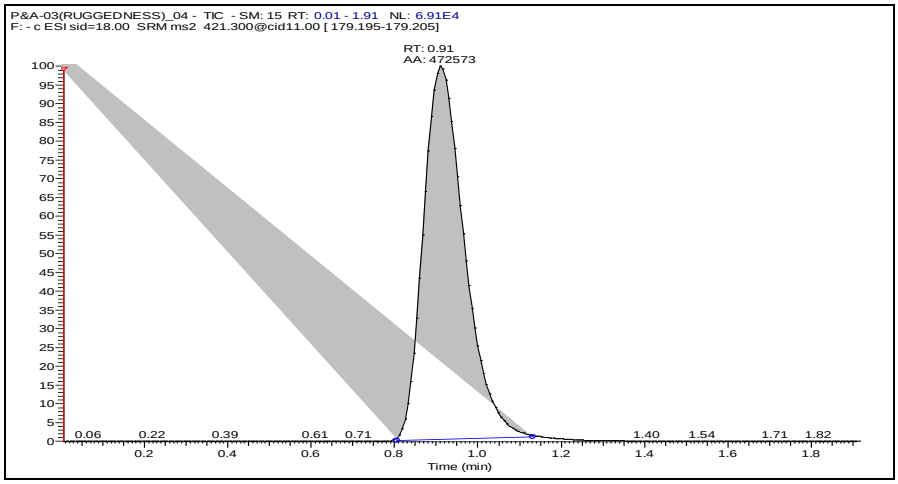


FIG. 2: SPECIFICITY AND SELECTIVITY

TABLE 3: PREPARATION OF SPECIFICITY AND SELECTIVITY

		Analyte-Nitrofurantoin			ISTD-Losartan		
S. no	Plasma Lot No.	Area of interfering peak at RT of Analyte	Area observed for extracted LLOQ	% interference at RT of Analyte	Area of interfering peak at RT of ISTD	Area observed for extracted ISTD	% interference at RT of ISTD
1	LOT 1	0	7018	0.0	0	646317	0.0
2	LOT 2	0	7265	0.0	0	620841	0.0
3	LOT 3	0	6576	0.0	0	636653	0.0
4	LOT 4	0	6091	0.0	0	640451	0.0
5	LOT 5	0	6439	0.0	0	658280	0.0
6	LOT 6	0	6056	0.0	0	649608	0.0
	Mean		6574.1			642025.2	

Carry Over Effect: The sequence of injections containing two blank samples and two samples containing of LLOQ and ULOQ with internal standard were analyzed alternately to check if there is any carry over affecting the blank sample. There was no carryover effect observed in the present method.

Recovery: The peak areas of extracted QC samples were compared against the peak areas of respective aqueous QC samples. The % mean recovery for cycloserine in LQC, MQC and HQC was 79.38, 74.72 and 75.12% respectively. Recovery for IS was 73.28%. The results were shown in the **Table 4**.

Analyte (Nitrofurantoin) Recovery:

TABLE 4A: MQC CONCENTRATION LEVEL

QC-ID	Aqueous Area	Extracted Area
MQC	385414	381928
	395943	377500
	417814	347267
	350511	301452
	392824	338011
	401160	352982
Mean	390611.0	349856.7

TABLE 4B: HQC CONCENTRATION LEVEL

QC-ID	Aqueous Area	Extracted Area
HQC	761326	705893
	763073	695685
	753228	733564
	799654	785758
	784508	606553
	835182	693699
Mean	782828.5	703525.3

TABLE 4C: LQC CONCENTRATION LEVEL

QC-ID	Aqueous Area	Extracted Area
LQC	82716	78898
	77079	78586
	88886	83564
	82512	57957
	71351	65243
	72561	69086
Mean	79184.2	72222.3

TABLE 4D: TOTAL RESULTS OF ANALYTE RECOVERY

HQC	89.9
MQC	89.6
LQC	93.0
Average	90.8333
SD	1.8823
%CV	2.06

TABLE 5A: INTERNAL STANDARD (LOSARTAN) RECOVERY

	Aqueous Area	Extracted Area
HQC	271130	276519
	276010	277961
	267479	291076
	270622	277356
	281986	273247
MQC	281459	300743
	275058	285905
	266841	288092
	285684	317747
	247565	306391
LQC	271793	251363
	277453	238767
	271299	257841
	265772	274409
	295935	280751
	274500	286071
	239484	283411
	240319	265221
Mean	270021.6000	279603.9000
SD	14727.6700	19021.1300
% CV	5.5	6.8
% Recovery	103.5	

TABLE 5B: TOTAL RESULTS OF ANALYTE RECOVERY

HQC	89.9
MQC	89.6
LQC	93.0
Average	90.8333
SD	1.8823
%CV	2.06

Linearity, Accuracy, Precision and Sensitivity: The calibration curve was constructed using 9 calibration standards ranging from 10.45ng/ml to 1033.897ng/ml. a straight line fit was made through the data points by $1/x^2$ weighing method. The correlation coefficient was found to be ≥ 0.99. The lower limit of quantification (LLOQ) was found to be 10.516ng/ml. The sensitivity results were shown in following **Table 7**. The percent accuracy observed for the mean of back calculated concentrations for four calibration curves for nitrofurantoin was within 97.8-109.7, while % CV values ranged from 4.8-10.9. Accuracy and precision for intraday and inter day plasma homogenate samples are presented in the **Table 6, 7** and **8**. The following chromatograms represents accuracy and precision for LQC, GMQC, MQC and LQC samples. (**Fig. 3, 4, 5** and **6**).

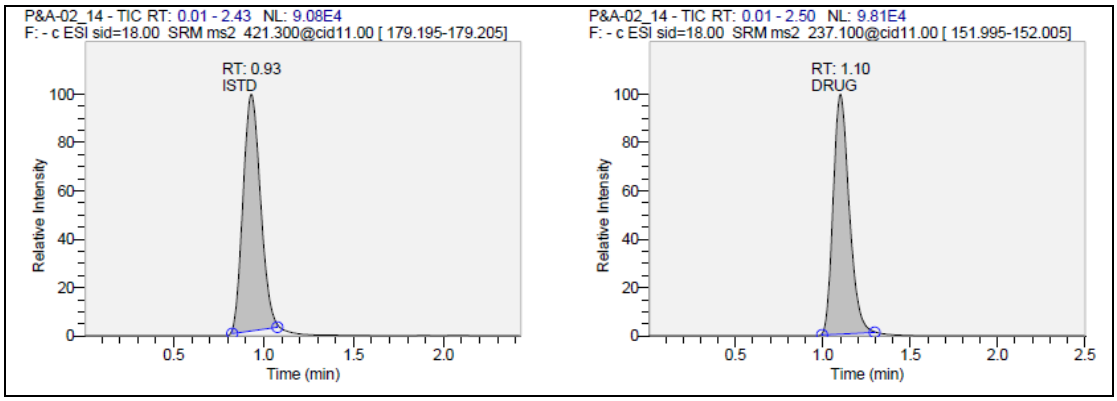


FIG. 3: CHROMATOGRAM OF HQC LEVEL CONCENTRATION OF NITROFURANTOIN

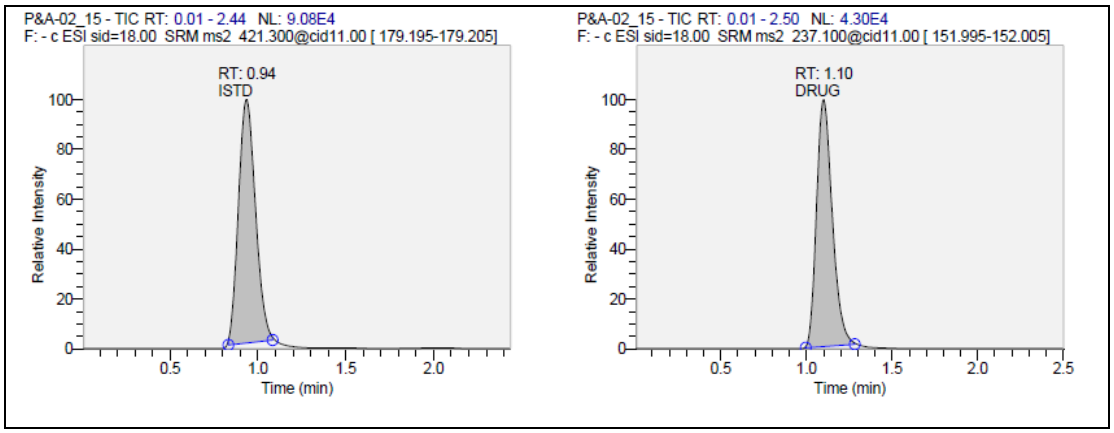


FIG. 4: CHROMATOGRAM OF MQC LEVEL CONCENTRATION OF NITROFURANTOIN

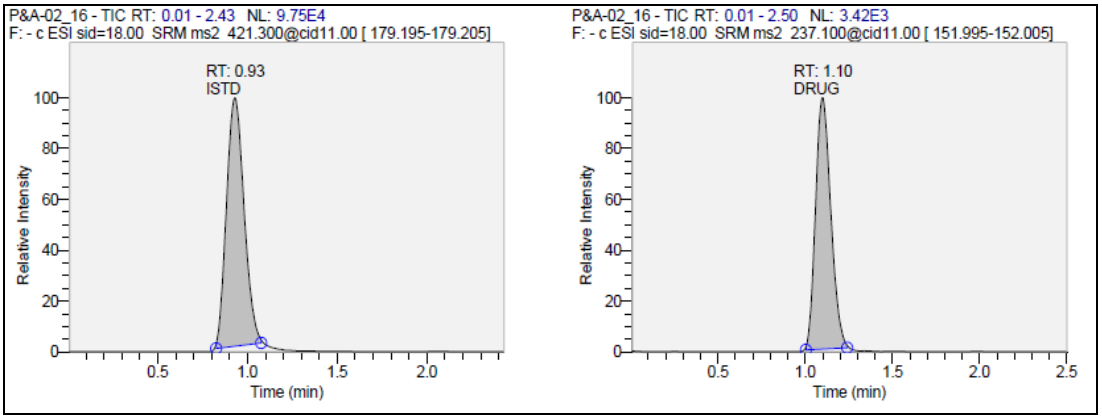


FIG. 5: CHROMATOGRAM OF LQC LEVEL CONCENTRATION OF NITROFURANTOIN

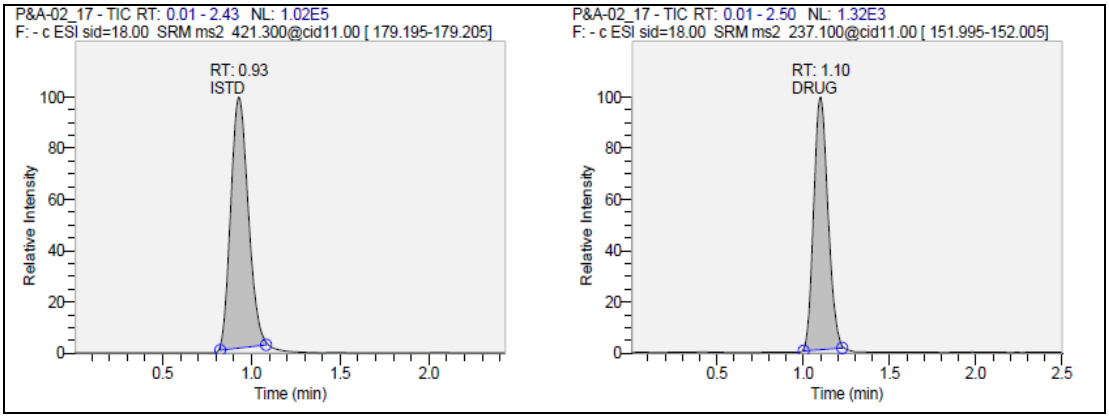


FIG. 6: CHROMATOGRAM OF LLOQ QC LEVEL CONCENTRATION OF NITROFURANTOIN

TABLE 6: SENSITIVITY OF NITROFURANTOIN

Actual Concentration (ng/ml)	**LLOQ 10.516**
1	11.792
2	11.417
3	11.357
4	12.141
5	11.605
6	10.522
Mean	11.4724
SD	0.5452
%CV	4.8
% Accuracy	109.1

TABLE 7: RESULTS OF PRECISION AND ACCURACY WITHIN BATCH OF NITROFURANTOIN

QC-ID	**HQC**	**MQC**	**LQC**	**LLOQ**
Actual concentration (ng/ml)	878.812	364.707	28.812	10.516
	902.132	395.541	29.365	8.929
	817.447	325.615	28.952	33.865
	748.133	321.296	30.071	8.692
	964.761	359.552	25.934	8.500
	787.459	336.101	29.036	8.489
	937.105	336.415	26.939	8.761
Mean	859.5060	345.7530	28.3830	8.6740
SD	87.5000	27.7610	1.5900	0.1850
%CV	10.2	8.0	5.6	2.1
% Accuracy	97.8	94.8	98.5	82.5
	882.722	363.175	27.189	11.550
	890.648	330.480	31.236	11.835
	889.838	347.524	36.009	12.450

	1154.519	375.567	31.770	13.037
	872.328	352.082	32.880	12.079
	1069.678	347.941	30.377	12.439
Mean	959.9550	352.7950	31.5770	12.2320
SD	121.0440	15.3370	2.9050	0.5260
% CV	12.6	4.3	9.2	4.3
% Accuracy	109.2	96.7	109.6	116.3
	858.366	366.757	28.885	11.792
	892.020	329.451	32.345	11.417
	849.483	342.189	35.243	11.357
	1071.416	375.739	32.018	12.141
	867.743	371.061	32.716	11.605
	1052.868	354.862	28.398	10.522
Mean	931.9830	356.6760	31.6010	11.4720
SD	101.9850	18.0350	2.5640	0.5450
% CV	10.9	5.1	8.1	4.8
% Accuracy	106.1	97.8	109.7	109.1

TABLE 8: RESULTS OF PRECISION AND ACCURACY BETWEEN BATCHES OF NITROFURANTOIN

QC-ID	HQC	MQC	LQC	LLOQ
Actual concentration (ng/ml)	878.812	364.707	28.812	10.516
	902.132	395.541	29.365	8.929
	817.447	325.615	28.952	*33.865
	748.133	321.296	30.071	8.692
	964.761	359.552	25.934	8.500
	787.459	336.101	29.036	8.489
	937.105	336.415	26.939	8.761
	902.132	395.541	29.365	8.929
	817.447	325.615	28.952	*33.865
	748.133	321.296	30.071	8.692
	964.761	359.552	25.934	8.500
	787.459	336.101	29.036	8.489
	937.105	336.415	26.939	8.761
	858.366	366.757	28.885	11.792
	892.020	329.451	32.345	11.417
	849.483	342.189	35.243	11.357
	1071.416	375.739	32.018	12.141
	867.743	371.061	32.716	11.605
	1052.868	354.862	28.398	10.522
Mean	883.6650	349.3940	29.4550	9.7230
SD	93.8012	24.0222	2.4203	1.4404
% CV	10.61	6.87	8.21	14.81
% Accuracy	100.55	95.8	102.22	92.48

Stability: The predicted concentrations for nitrofurantoin at LQC and HQC samples deviated with in ±15% of nominal concentration in stability tests *i.e.* auto sampler stability (36 hrs), bench top (6 hrs), repeated three freeze thaw cycles, 24hrs of dry extract stability. The results were found to be within the acceptance limits during the entire process (**Table 9**).

TABLE 9: FREEZE THAW STABILITY FOR NITROFURANTOIN QUALITY CONTROL SAMPLES QUALITY CONTROL SAMPLES

	Freshly spiked		FT Cycle III	
	LQC	HQC	LQC	HQC
	Actual concentration (ng/mL)			
S. No.	27.892	850.765	28.812	878.812
1	27.892	850.765	30.932	894.515
2	28.766	777.603	25.774	887.512
3	30.425	726.666	30.870	878.819
4	31.703	858.704	26.016	936.995

5	33.174	807.561	28.757	870.821
6	40.189	829.280	32.495	748.152
MEAN	32.0247	808.4298	29.1405	869.4692
SD	4.4348	49.7810	2.7820	63.7489
% CV	13.4	6.0	9.5	7.3
		% Stability	88.1	104.1

TABLE 10: BENCH- TOP STABILITY FOR NITROFURANTOIN QUALITY CONTROL SAMPLES

	Freshly spiked		**6 hours**	
	LQC	**HQC**	**LQC**	**HQC**
	Actual concentration (ng/mL)			
S. No.	**27.892**	**850.765**	**28.812**	**878.812**
1	27.892	850.765	28.812	878.812
2	28.766	777.603	25.374	545.275*
3	30.425	726.666	32.914	912.576
4	31.703	858.704	25.100	801.950
5	33.174	807.561	31.588	805.710
6	40.189	829.280	28.093	799.702
Mean	26.1560	745.4630	29.3480	811.0080
SD	4.4348	49.7810	3.1776	52.4902
% CV	16.4	6.5	10.8	6.5
		% Stability	108.6	105.3

TABLE 11: AUTO SAMPLER STABILITY FOR NITROFURANTOIN QUALITY CONTROL SAMPLES

	Freshly spiked		**36 hours**	
	LQC	**HQC**	**LQC**	**HQC**
	Actual concentration (ng/mL)			
S. No.	**27.892**	**850.765**	**28.812**	**878.812**
1	27.892	850.765	34.866	827.089
2	28.766	777.603	31.083	608.694
3	30.425	726.666	25.977	734.222
4	31.703	858.704	32.427	767.973
5	33.174	807.561	30.379	758.263
6	40.189	829.280	33.674	774.536
Mean	32.0247	808.4298	31.4009	745.1297
SD	4.4348	49.7810	3.1240	73.4985
% CV	13.4	6.0	9.9	9.9
		% Stability	94.9	89.2

TABLE 12: DRY EXTRACT STABILITY FOR NITROFURANTOIN QUALITY CONTROL SAMPLES

	Freshly spiked		**24hours**	
	LQC	**HQC**	**LQC**	**HQC**
	Actual concentration (ng/mL)			
S. No.	**27.892**	**850.765**	**28.812**	**878.812**
1	27.892	850.765	28.373	766.546
2	28.766	777.603	27.658	*746.073
3	30.425	726.666	18.716	*525.883
4	31.703	858.704	34.067	*593.530
5	33.174	807.561	24.040	904.203
6	40.189	829.280	30.853	839.989
Mean	32.0247	808.4298	27.2846	836.9126
SD	4.4348	49.7810	5.3669	68.8797
% CV	13.4	6.0	19.7	8.2
		% Stability	82.5	100.2

CONCLUSION: A LC-MS/MS method was developed for the determination of nitrofurantoin in human plasma by using simple liquid - liquid extraction method. The method is simple, rapid, selective, specific, shows good accuracy and precision and cost effective With less run time 2.5 min and ability to quantify the drug in Nanogram level This method can be used for routine analysis

of determination of nitrofurantoin in human plasma by using LC-MS/MS method

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CONFLICTS OF INTEREST: Nil.

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