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A NOVEL METHOD FOR THE DETERMINATION OF SECNIDAZOLE IN HUMAN PLASMA BY USING LIQUID CHROMATOGRAPHY-ELECTRO SPRAYS IONIZATION TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: A novel rapid, specific and sensitive liquid chromatography tandem mass spectrometry (LCMS/MS) method was developed for the determination of Secnidazole in K_2EDTA human plasma. **Methods:** The method involves simple, liquid-liquid extraction procedure and separation with an Inertsil® ODS-3V (5µm, 4.6 × 150 mm) with Acetonitrile / 10mM Ammonium acetate [70/30, V/V] isocratic elution at a flow-rate of 1.0 mL/min with a total run time of 2.50 minutes. Labeled isotope Secnidazole D6 was used as the internal standard. The protonate of analyte's were quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions m/z 185.95 →127.73 and m/z 192.04 →127.76 were used to measure Secnidazole and Secnidazole D6 respectively. **Results:** The method was developed and validated using 100 µL of plasma, over a concentration range of 0.200 – 40.032 µg/mL. The intra and inter day Precision and Accuracy values were found <3.77 and 103.48%. The overall extraction efficiency was observed 66.79%. All the stability parameters were within the assay variability limits as per regulatory guidelines. **Conclusion:** This is a novel and sensitive and cost effective method. Total run time was 2.50 min only for each sample analysis. By using this method 250 samples analyzed per day. The method can be successfully applied to a human pharmacokinetic study and has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring

KEYWORDS: Secnidazole, tandem mass spectrometry, HPLC, LC-MS/MS, Human plasma.

INTRODUCTION

Secnidazole is chemically known as (1-(2-Methyl-5-nitroimidazol-1-yl) propan-2-ol). Secnidazole is not official in any pharmacopoeia. A nitroimidazole anti-infective. Effectiveness in the treatment of dientamoebiasis.[1] Antiprotozoal and antiamoebic activity of secnidazole is by nitro group of nitro imidazole is chemically reduced by ferrodoxin which is ferrodoxin-Linked Metabolic process. After entering into the microorganism by diffusion, its nitro group is reduced to intermediate compound which cause cytotoxicity, by damaging DNA. Its selectivity high activity against anaerobic organism has suggested interference with electron transport from NADPH or other reduced substrate.[2]

Very few analytical methods have been reported for the determination of Secnidazole includes few methods based on techniques viz. colorimetric,[3] UV Spectrophotometric[4-6] and RP-HPLC[7-9] for its determination in pharmaceutical dosage form and in human plasma. But no LCMS/MS method has so far been reported for the determination of secnidazole in pharmaceutical preparations as well as biological fluids. So this is only the novel method for estimation of secnidazole in single run by using LC-ESI-MS/MS with major regulatory guidelines.[10-15]

MATERIAL AND METHODS

Reference standards, Chemicals and Reagents

Secnidazole {chemical purity 98.06%, $C_7H_{11}N_3O_3$, MW= 185.180 g/mol} and its Internal standard Secnidazole D6 {chemical purity 98.70%, $C_7H_5D_6N_3O_3$, MW= 191.22 g/mol} (Fig. 1 A & B) were obtained from Shubam biopharma (Mumbai, India). HPLC grade solvents Acetonitrile, Methanol, Ethyl acetate, Ammonia solution, Water and Ammonium acetate were Merck products (Merck, India). Human plasma with K_2EDTA as anticoagulant was obtained from in-house clinical facility of Aizant Drug Research Solutions Pvt ltd.

Instrumentation

Liquid chromatography with tandem mass spectrometry detection was performed on Waters QuatrropremeirXE triple quadruple mass spectrometer equipped with electro

spray ionization (ESI) probe interfaced to a separation module alliance 2695 HPLC system from waters.

Chromatographic condition

A alliance 2695 HPLC system, consisting of an auto sampler, a multichannel mobile phase degasser, a column heater, four pumps and a Inertsil® ODS-3V (5µm, 4.6 × 150 mm) (GL sciences, India), was used for the chromatographic separation of Secnidazole and its internal standard. The mobile phase used was Acetonitrile / 10mM Ammonium acetate [70/30, V/V]. Flow rate was set to 1.00 mL/min with 30% flow splitting to the mass spectrometer. Column oven temperature was 40 ± 5ºC and auto sampler temperature was 5 ± 3ºC. Volume of injection was 10 µL and runtime was 2.50 minutes.

Mass spectrometric conditions

Analyte's were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of precursor-product ion transitions with 100 ms dwell time, at m/z 185.95/127.73 for Secnidazole, m/z 192.04/127.76 for Secnidazole D6. The instrument dependent parameters were optimized and maintained as follows: capillary voltage 3.50 kV; Extractor 3.0 v; source temperature 120ºC, Desolvation Temperature 450ºC, Desolvation gas flow 900 L/Hr. Compound dependent parameters like Cone 15, collision energy 15 for secnidazole and secnidazole D6. Data acquisition and processing were performed using Mass lynx software, Version 4.1.

Preparation of stock solution, standard and quality control samples

Stock solutions of Secnidazole and Secnidazole D6 were prepared by dissolving accurately weighed standard compounds in methanol to yield a concentration of 5 mg/mL. All subsequent dilutions were made with methanol/water 50/50 v/v. Standard working solutions at concentrations of 0.200, 0.400, 2.002, 4.003, 10.008, 20.016, 32.026 and 40.032 µg/mL. were prepared by serial dilutions. QC working solutions at concentrations of 0.200, 0.565, 1.766, 3.532, 18.591, 30.476 and DQC 95.239 µg/mL were also prepared by successively diluting the 5mg/mL QC stock solution.

The internal standard stock solution was diluted to a working concentration of 10 µg/mL. These working solutions were stored at 2-8ºC. The linearity curve was built by 2% spiking of drug into the screened human plasma.

Liquid-Liquid Extraction (LLE) procedure

Extracted Sample Preparation

Plasma samples frozen at below -25ºC were thawed at room temperature followed by vortexing to ensure homogeneity. For the determination of Secnidazole, 50 µL of ISTD working solution was transferred to polypropylene tubes followed by 100 µL of spiked plasma and vortexed for 5 seconds. To this 100 µL of extraction buffer (0.1% Ammonia) was added and vortexed for about 10 seconds, then added 1.0mL of Organic solvent (Ethyl acetate) to all the tubes, vortexed for 10 min, centrifuged, collected 0.800 mL of organic solvent and evaporated to dryness under nitrogen gas at 40 ± 5°C using TurboVap (Caliper life sciences, United States). Finally 0.600 mL of reconstitution solution was added to all the tubes and vortexed for about 1 minute. An appropriate volume of the reconstituted solution was transferred into pre-labeled autosampler vials and injected 10µL into LC-MS/MS.

Aqueous Sample Preparation

About 500 µL of mixed ISTD working solution were added into pre-labeled tubes. To this 20µL of respective spiking solution was added and vortexed, followed by 5480 µL of reconstitution solution and vortexed. An appropriate volume of the reconstituted solution was transferred into pre-labeled autosampler vials and 10µL was injected into LC-MS/MS.

Method validation

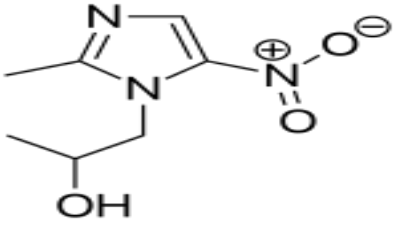
A full Method validation was performed according to guidelines set by US FDA (33). The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity of response, accuracy, precision, recovery, matrix effect and matrix factor, ruggedness, reinjection reproducibility, effect of potential interfering drugs, stability of analytes during both short-term sample processing and long-term storage.

RESULTS AND DISCUSSION

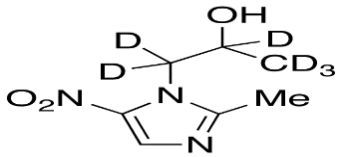
LC-MS/MS condition optimization

The product ion spectra of Secnidazole and its internal standard were obtained (Fig. 2 A to B). Several fragment ions were observed in the product ion spectra for Secnidazole. The fragment ion at m/z 127.73 was chosen as product ion for Secnidazole, m/z 127.76 for Secnidazole D6. As these ions presented a higher abundance and stability with no cross-talk effect.

The composition of mobile phase includes acetonitrile/ 10mM Ammonium acetate. Lesser mM of ammonium acetate and less acidic condition was chosen in the mobile phase to increase the sensitivity and for better peak shape. The retention time of Secnidazole was 1.70 minutes. A representative chromatogram of double blank (A), standard Zero (B), and lower limit of quantitation (LLOQ) (C) and upper limit of quantitation (ULOQ) (D) samples were summarized in (Fig. 3).



A. Structure of Secnidazole.



B. Structure of Secnidazole D6.

Fig. 1: Legends of figures.

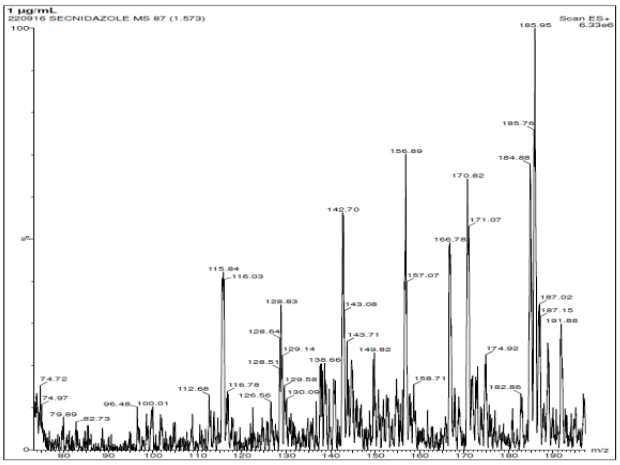


Fig. 2: A. MS Spectrum of Secnidazole. B. MS Spectrum of Secnidazole D_6

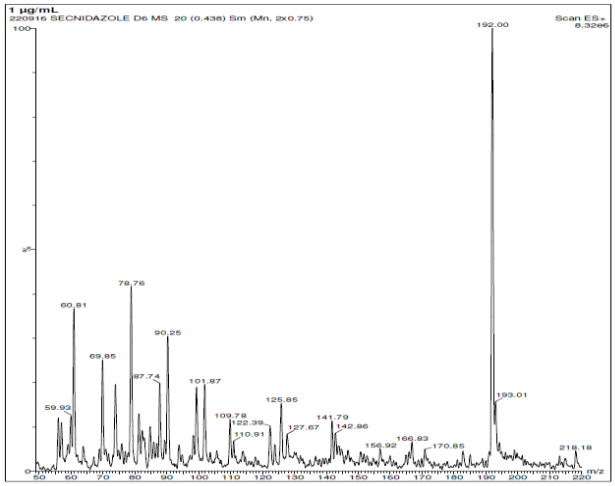
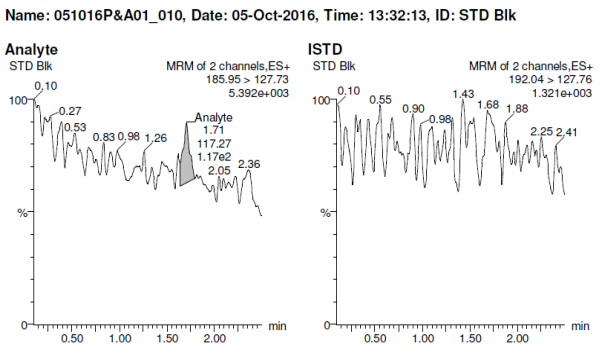
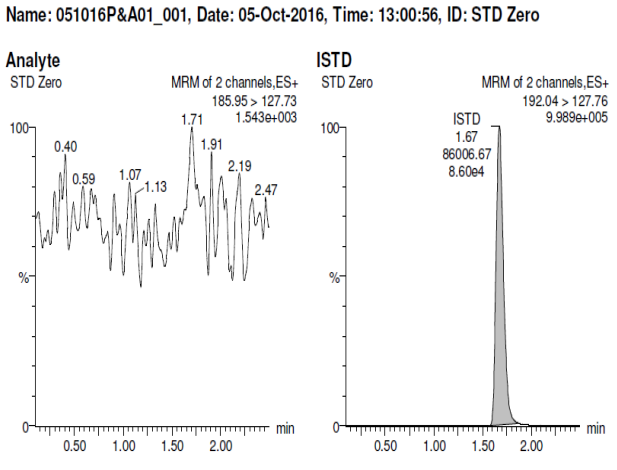


Fig. 3: A. Chromatograms of STD Blk for Secnidazole, Secnidazole D6.



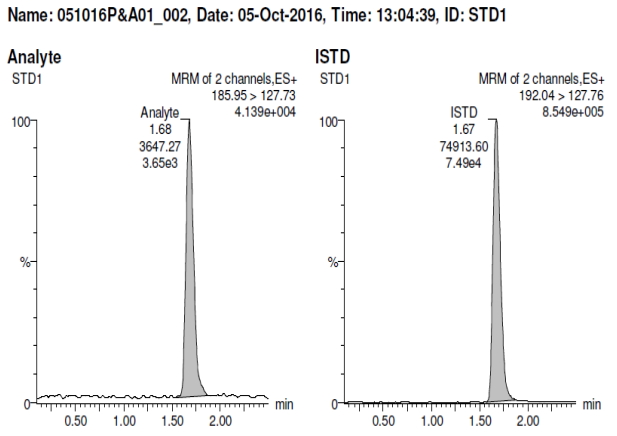
Name	RT	Area	IS Area	Area Ratio	µg/mL	Detection Flags
Analyte	1.71	117				db
ISTD						

C. Chromatograms of STD Zero for Secnidazole, Secnidazole D6.



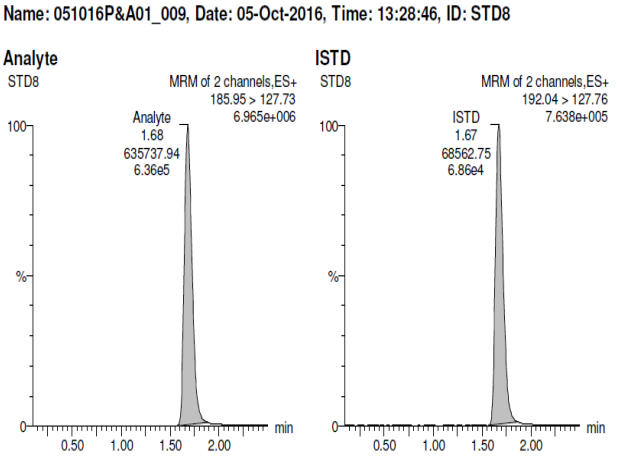
Name	RT	Area	IS Area	Area Ratio	µg/mL	Detection Flags
Analyte			86007			
ISTD	1.67	86007	86007	1.0000	1.0	bb

D. Chromatograms of LLOQ for Secnidazole, Secnidazole D6.



Name	RT	Area	IS Area	Area Ratio	µg/mL	Detection Flags
Analyte	1.68	3647	74914	0.0487	0.2	bb
ISTD	1.67	74914	74914	1.0000	1.0	bb

E. Chromatograms of ULOQ for Secnidazole, Secnidazole D6.



Name	RT	Area	IS Area	Area Ratio	µg/mL	Detection Flags
Analyte	1.68	635738	68563	9.2724	39.5	bb
ISTD	1.67	68563	68563	1.0000	1.0	bb

Table 1: Precision and accuracy of Secnidazole.

Nominal concentration of Secnidazole (µg/mL)	Intra-day (n=6)			Inter-day (n=6)		
	Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
HQC (30.476)	29.8745	1.04	98.03	29.3832	1.43	96.41
MQC1 (18.591)	18.3068	1.01	98.47	17.9452	1.71	96.53
MQC2 (3.532)	3.7185	1.47	105.28	3.6191	2.39	102.47
MQC3 (1.766)	1.7752	0.96	103.07	1.7944	1.69	101.61
LQC (0.300)	0.5407	1.48	97.03	0.5417	1.97	98.88
DQC (95.239)	99.1150	3.51	104.07	98.5509	2.24	103.48
LLOQ (0.200)	0.2032	1.74	101.60	0.2044	3.77	102.20

Table 2: Recovery of analyte and ISTD.

Analyte	HQC	MQC1	LQC	Over all mean accuracy	CV (%)
Secnidazole	67.40	65.97	67.10	66.79	1.21
Secnidazole D6	69.11	68.70	69.28	69.03	0.43

Table 3: Matrix effect.

	HQC	LQC
Overall mean (%)	1.036	0.994
CV (%)	2.58	0.93

Table 4: Stability data of Secnidazol.

Name of the Experiment	Condition	Stability Period
Freeze Thaw Stability	-28 ± 5 °C	5 Cycles
	-70 ± 10 °C	5 Cycles
Bench Top Stability	Room Temperature	06 hours 42 minutes
Auto sampler Stability	5±3°C	67 hours 33 minutes
Wet Extract Stability	Room Temperature	17 hours 37 minute
	2-8 °C	67 hours 24 minutes
Dry Extract Stability	Room Temperature	17 hours 42 minutes
Blood Stability (Analyte)	Room Temperature	03 hours 13 minutes
	2-8 °C	03 hours 13 minutes
Short Term Stock Solution Stability for Analyte's & ISTD's	Room Temperature	06 hours 07 minutes
Short Term Spiking/Working Solution Stability for Analyte's & ISTD's	Room Temperature	06 hours 05 minutes
Long Term Stock Solution Stability for Analyte & ISTD	2-8 °C	08 days 20 hours
Long Term Spiking/Working Solution for Analyte & ISTD	2-8 °C	08 days 18 hours
Long Term stability in Matrix	-28 ± 5 °C	36 days
	-70 ± 10 °C	36 Days

Sample preparation optimization

Protein precipitation, liquid-liquid extraction and Solid phase extraction are often used in preparation of biological samples due to their ability to improve the sensitivity and robustness of assay. Due to interference peaks observed in protein precipitation extraction method was developed in liquid-liquid extraction technique. In LLE technique extraction buffer and organic solvents plays a major role for their selectivity and extraction issues. As the alkaline condition was useful for the good recovery of the analyte. For extraction purpose we tried ammonium acetate p^H 2.50, 0.1%Formic acid, 0.1% Ammonia solution, finally 0.1% Ammonia solution was chosen because of better recovery. For eluting the interference peaks different organic solvents were tried and finally chosen medium polar organic solvent. The Optimization of these parameters in the LLE technique made the method more sensitive, rugged, no matrix interferences and good recoveries.

Method validation parameters

Carryover Effect

The carryover effect due to the auto sampler was investigated by injecting a sequence of unextracted samples consisting of RS, AQ ULOQ, RS, RS, AQ LLOQ and extracted samples containing STD Blk, ULOQ, STD Blk, STD Blk and LLOQ. No significant carry over observed during this experiment.

Linearity and Sensitivity

The linearity of the method was determined (in K_2EDTA) by using a $1/x^2$ weighted least square regression analysis of standard plots associated with an Ten-point standard curve. All the three calibration curves analyzed during the course of validation were found to be linear. The correlation coefficient (r) was observed to be ≥ 0.9993 during the course of validation.

The Sensitivity of the method was evaluated by analyzing six LLOQ samples. The % CV and % mean accuracy at LLOQ level were found 1.74 and 101.60 for Secnidazole.

The S/N ratio Calculated for sensitivity experiment was found to be more than 197.

Precision and accuracy

The precision (% CV) of the LC-MS/MS method was evaluated in K_2EDTA by analyzing 6 replicates at different concentration levels corresponding to HQC, MQC1, MQC2, MQC3, LQC, DQC and LLOQ during the course of validation.

Within Batch Precision and accuracy

The % CV of back calculated concentrations for all quality control samples of HQC, MQC1, MQC2, MQC3, LQC and DQC concentration levels were ranged from 0.96 to 3.51. The % CV of back calculated concentration for all the samples of LLOQ was found to be 1.74.

The % mean accuracy of back calculated concentrations for all quality control samples at HQC, MQC1, MQC2, MQC3, LQC and DQC concentration levels were ranged from 97.03 to 105.28. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 101.60.

Between Batch Precision and accuracy

The % CV of back calculated concentrations for all quality control samples at HQC, MQC1, MQC2, MQC3, LQC and DQC concentration levels were ranged from 1.43 to 2.39. The % CV of back calculated concentration for all the samples of LLOQ was found to be 3.77.

The % mean accuracy of back calculated concentrations for all quality control samples at HQC, MQC1, MQC2, MQC3, LQC and DQC concentration levels were ranged from 95.88 to 103.48. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 102.20.

The results were summarized in table 1.

Recovery

Recovery for Analyte

The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1 and LQC levels. The % mean recovery at HQC, MQC1 and LQC levels was found to be 67.40, 65.87 and 67.10 respectively. Over all % mean recovery and % CV at all QC levels was found to be 66.79 and 1.21 respectively.

This is within the acceptance limit of 15.00 %.

Recovery for Internal Standard

The % mean recoveries were determined by measuring the responses of Secnidazole D6 in the extracted samples against unextracted samples at HQC, MQC1 and LQC levels respectively. The % mean recovery at HQC, MQC1 and LQC levels was found to be 69.11, 68.70 and 69.28 respectively. Over all % mean recovery and % CV at all QC levels was found to be 69.03 and 0.43 respectively.

Matrix Effect

Matrix effect was assessed by using 8 different lots (4 normal plasma, 2 haemolytic plasma and 2 lipemic plasma) of previously screened plasma lots. Blank samples in duplicate for each lot in each level were processed as per the respective method SOP, samples were spiked to achieve the concentration equivalent to HQC and LQC were injected. Un-extracted samples concentration equivalent to HQC and LQC were also prepared and injected. The % CV of ISTD normalized matrix factor at HQC and LQC samples were found to be 2.58 and 0.93 respectively. The results were summarized in table 3.

Ruggedness

Ruggedness was performed by analyzing one P&A batch using different column, different analyst and different equipment. The % CV of back calculated concentrations for all quality control samples were ranged from 0.53 to 2.14 and the % mean accuracy of back calculated concentrations for all quality control were ranged from 95.71 to 105.69.

Selectivity of Concomitant Drugs

Concomitant drugs was performed using 1 STD Blk by spiking concomitant spiking solution separately for each concomitant drug (Paracetamol, Caffeine, Diclofenac, Nicotine, Ondansetron, Pantoprazole and Hyoscine) and 3 samples equivalent to LLOQ using screened blank plasma and analyzed.

There was no effect observed by all the above drugs on Secnidazole.

Stability of Analytes

The stabilities of Secnidazole were investigated at two concentrations of QC samples (Low (LQC) and High (HQC) concentrations) to cover expected conditions during analysis, storage and processing of all samples. Stability was assessed by comparing the stability samples against the comparison samples with freshly prepared calibration curve. Which include the stability data from various stability exercise like auto sampler, dry extract, wet extract, bench-top, freeze thaw, blood, short term and long-term stability tests. These data were summarized in table 4.

CONCLUSION

A novel, simple, sensitive, accurate and reproducible LC-MS/MS method has been developed and validated for the estimation of Secnidazole in human plasma. This method was developed and validated using 100 µL of plasma, over a concentration range of 0.200 – 40. 032 µg/mL this is a novel, sensitive and less runtime method Total run time was 2.50 min only for each sample. By using this method 250 samples analyzed per day. All the stability parameters were within the assay variability limits as per regulatory guidelines. The method can be successfully applied to a human pharmacokinetic study and has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

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