



SEPARATION AND ASSAY OF ANTIPROTOZOAL IMIDAZOLE DERIVATIVES (METRONIDAZOLE, TINIDAZOLE AND SECNIDAZOLE) BY RP-HPLC

Zayan Alhalabi^{1*}, Mohamad Ammar Al-khayat², Samer Haidar³

¹ License in Pharmacy, Stage of preparing master degree in drug quality control, Faculty of Pharmacy, Damascus University, Damascus.

² Professor at the Dept of Pharmaceutical Chemistry and drug Quality Control, Faculty of Pharmacy, Damascus University, Damascus.

³ Assistant professor at the Dept of Pharmaceutical Chemistry and drug Quality Control, Faculty of Pharmacy, Damascus University, Damascus.

*Corresponding author's E-mail: zayan82at@hotmail.com

Accepted on: 06-01-2012; Finalized on: 20-02-2012.

ABSTRACT

A reverse phase high performance liquid chromatography method was developed in this research for separation and assay of antiprotozoal imidazole derivatives (Metronidazole, Tinidazole and Secnidazole). The separation was achieved by C8 column using acetonitrile: water (20:80 v/v) as a mobile phase, a flow rate of 1.5 ml/min. UV detection was carried out at 318 nm. The retention times of metronidazole, secnidazole and tinidazole were 4.140, 5.515 and 6.692 min respectively. The qualitative study of this method included the effect of flow rate and the ratio of the components of mobile phase on the quality of separation. The method was validated for accuracy, precision, selectivity and robustness. Linearity of metronidazole, secnidazole and tinidazole were in the range of 80-120 µg/ml. The relative standard deviation for precision was not more than 2%. The recoveries in accuracy obtained for metronidazole, secnidazole and tinidazole were 99.22%, 99.89 and 99.64% respectively. The developed method was found to be rapid, accurate and precise and was used for the determination of the three compounds in raw materials and tablets. This method was also used for the assay of these three compounds in the spiked human serum (1-30 µg/ml) and recoveries were in the range of 88-96%.

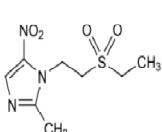
Keywords: RP-HPLC, Metronidazole, Tinidazole, Secnidazole.

1. INTRODUCTION

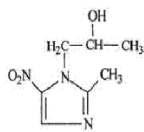
The history of Nitroimidazole begins in 1953, when antibiotic called azomycin (2 - Nitroimidazole) was isolated from *Streptomyces* species. It was found that this compound was active against protozoas, especially *Trichomonas vaginalis*.^{1,2}

In 1957 metronidazole was introduced as a drug of choice for the treatment of various protozoa including, *Giardia lamblia* and *Trichomonas vaginalis*, and since then, researchs were focused on the synthesis of several derivatives of Nitroimidazole, and this led to the synthesis of a new antiprotozoal compounds such as Tinidazole and Secnidazole.²

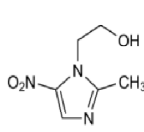
The drug substances studied in this research are metronidazole, Tinidazole and Secnidazole. They are derivatives of 5 - Nitroimidazole (Figure 1) they possess antiprotozoal activity and anti-bacterial activity for gram negative and Gram-positive anaerobic bacteria. It is used in the human and veterinary medicine.^{3,4}



Tinidazole



Secnidazole



Metronidazole

Figure 1: Chemical Structures of metronidazole, Secnidazole and Tinidazole^{5,6}

Pharmacopieas (BP 2009, Eur 2009) mention volumetric non-aqueous titration for assay of metronidazole or tinidazole in raw material or tablet while USP 2009 mentions HPLC method with C8 column for assay of metronidazole in tablet.^{5,7,8}

International references mention various analytical methods for the determination of metronidazole, Tinidazole and Secnidazole in pharmaceutical preparations and human serum it is include: volumetric⁹ spectrophotometric,¹⁰⁻¹³ GC-FID¹⁴, HPLC¹⁵⁻²² and polarograph²³.

Variety of 5-nitroimidazole mixtures were also analyzed in meat, eggs and animal feed, methods used for this purpose include: HPLC-UV²⁴, LC-MS^{25,26}, supercritical fluid chromatography²⁷ and gas chromatography GC-MS^{28,29}. Our mixture studied in tablet in this research is not mention in previously papers.

The aim of this study was to develop a valid, rapid and sensitive analytical procedure using high performance liquid chromatography (HPLC) for the separation and assay of a mixture of metronidazole and Tinidazole and Secnidazole in tablets, Attempts were also done to determine these three drugs in spiked serum in the range of 1-30 µg/ml.

2. MATERIALS AND METHODS

Reference standards of metronidazole, tinidazole and secnidazole were obtained from the Syrian health Ministry.

The samples of raw materials were obtained from several local private pharmaceutical factories, pharmaceutical tablets sample were purchased from the Community Pharmacy.

Human serum samples were obtained from a hospital laboratory.

2.1. Chemicals and solvents

- Potassium dihydrogen phosphate was purchased from Riedel De Hean Company.
- HPLC Solvents (Acetonitrile, Methanol, water) were purchased from the Merck, Germany.
- pH solutions: Hana company, Hungary.

2.2 Instrument and Equipments

JASCO high pressure liquid chromatography system provided with two pump (PU-980 Intelligent HP) and UV / VIS detector (UV-970), and manual injector (20 µl loop)

- An Ultrasonic device T310 Germany
- pH meter model Orion 410 A
- magnetic stirrer model Labinco L33
- Filters 0.45 µm from Whatman Inc.

HPLC filters 0.45 µm from Sartorius stedium biotech company.

C8 column (250X4.6) mm Eurospher, 5µm from -Knauer Germany.

Sensitive balance Sartorius TE64 model (sensitivity of 0.0001 g)

- volumetric flasks, Pipette and glass of different sizes.

Centrifuge type HERMLE Z230A - cartridge 18: RP Adsorbex from Darmstadt. F.R. Merck Company for serum sample.

2.3. Preparation of solutions

Preparation of stock solution of metronidazole, secnidazole and tinidazole (1 mg / ml)

Weigh 100 mg standard of metronidazole or Tinidazole or Secnidazole, and place it in a 100 ml volumetric flask, dissolve it by an amount of acetonitrile, stir using a magnetic stirrer for 15-30 minutes until it is completely dissolved. Complete the volume with acetonitrile, a solution of concentration 1 mg / ml is obtained.

Preparation of Standards (0.1 mg / ml)

Take 10 ml by calibrated pipette from the stock solution 1 mg / ml of metronidazole or secnidazole or tinidazole and place it in a 100 ml volumetric flask. Complete volume of

the solution with the mobile phase to get the concentration of 0.1 mg / ml (100 µg / ml).

Preparation of validity test Solutions

• Standard linearity solutions

Prepare five sequential concentrations from the stock solution containing respectively 80%, 90%, 100%, 110% and 120% of the standard solution concentration of metronidazole or secnidazole or tinidazole. Put sequentially and separately 8, 9, 10, 11, 12 ml of the stock solution, in five volumetric flasks (100 ml).

Complete volume of the solution with mobile phase to get solutions of concentrations 80-90-100-110-120 µg / ml respectively, inject 20µl from each solution in the HPLC system and record the results.

• Accuracy solutions

Tablet excipients (Sorbitol, Kollidon CL, Talc, Aerosil 200, Calcium arachinate)³⁰ are added to the standard solutions of metronidazole or secnidazole or tinidazole.

Nine samples are divided into three groups containing respectively 80%, 100% and 120% of standard solution concentration.

Take at each time an amount equal to the weight of excipient in one tablet and place it in 100 mL volumetric flask, add 8 or 10 or 12 ml of stock solution, complete volume of the solution by the mobile phase and mix. The concentration of the active substance in the three groups respectively are 80, 100, 120 µg /ml.

• Repeatability solutions

Tablet samples are analyzed. Prepare nine samples and divide them into three groups containing respectively 80%, 100% and 120% of standard solution concentration of metronidazole and Tinidazole and Secnidazole.

For each sample of nine samples, weight of the amount of powder equivalent to 100 mg of the active substance and put it in a 100 ml volumetric flask and dissolve by an amount of acetonitrile then stir using a magnetic stirrer for a period of 15-30 minutes. Complete the volume of the solution by the acetonitrile, filter the sample by 0.45µ filters. Solution of concentration 1 mg / ml is obtained.

Take 8 ml or 10 ml or 12 ml and place in a 100 ml volumetric flask and then complete volume of the solution by the mobile phase then mix. Solutions of active substance with concentrations 80-100-120 µg /ml are obtained respectively.

• Intermediate precision solutions

Samples are prepared in the same way mentioned in the repeatability solutions. Assay is carried out after two weeks in the same experimental conditions.

Selectivity solution

A drug-free sample (excipients only) is prepared in acetonitrile; three samples containing excipient and



active ingredient are prepared in 100% standard solution concentration (100 µg/ml).

• Robustness solutions

Three tablet samples containing 100% of standard solution concentration are analyzed. The sample is injected at different flow rates 1.4, 1.5, and 1.6 ml/min.

Preparation of the standard solution mixture of metronidazole, Secnidazole and Tinidazole

Take 10 ml of each of the stock solutions of metronidazole, Secnidazole and Tinidazole. Place all in a 100 ml volumetric flask, complete the volume of the solution with the mobile phase. Stir using ultrasonic device for 15 minutes to get a solution of 100 µg/ml of metronidazole, Secnidazole and Tinidazole.

Preparation of samples

• Raw materials

Preparing the raw material sample of concentration 100 µg/ml in acetonitrile.

• Tablets

Prepare three tablet samples solutions in acetonitrile with concentration of 100% of standard solution of metronidazole or Tinidazole or Secnidazole, filter the solution prior to injection in a HPLC.

Preparation of Standard Solutions Series for Determination the Recovery from the Serum

Series of standard solutions were prepared in the following concentrations (1, 5, 10, 20, 30 µg/ml) of the three compounds by diluting their stock solutions 1mg/ml.

Preparation of Series of Serum Standard Solutions

The above-mentioned series of standard solutions were prepared with duplicated concentrations. 1 ml of series solutions is added to 1 ml drug-free serum to obtain a new series of serum standard solutions with the same concentrations of the previous series. Centrifuge for half an hour.

Extraction

Extraction was done by liquid/solid extraction method using C18 cartridge in the following way:

- The cartridge is preconditioned with 3 ml of methanol and withdrawn then with 3 ml of water and withdrawn.
- Serum sample is applied on the solid phase.
- Compounds were eluted with 10 ml acetonitrile, the elutes were left to dry for 15 minutes. Dry residues were dissolved with mobile phase. The solutions were run through 0.45 µm HPLC filters then injected directly to HPLC.

2.4. Chromatographic Conditions

Mobile phase: acetonitrile: water (20: 80) v / v

Flow rate: 1.5 ml / min

UV-detector: 318 nm

Column: C8, Eurospher (250X4.6) mm, 5µm Column

Temperature: 25°C

Injection volume: 20 µl

2.5. Method development

• Wavelength selection

Figure 2 shows the UV spectra of metronidazole, Tinidazole and Secnidazole³¹ wavelength 318 nm was selected for assay of these three compounds.

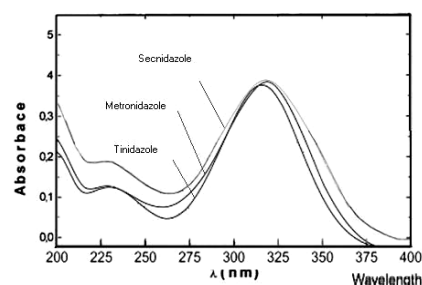


Figure 2: UV spectra of metronidazole and Secnidazole and Tinidazole

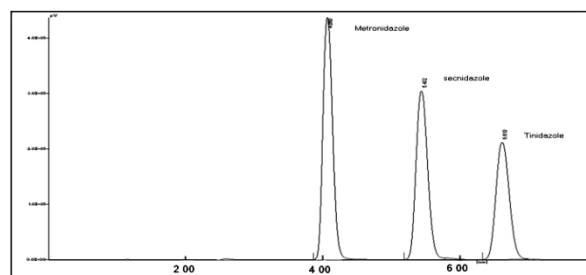


Figure 3: Chromatogram of metronidazole, Tinidazole and Secnidazole.

• A sample of a mixture of the three compounds was prepared in acetonitrile and injected into the HPLC system using the following experimental conditions: Column C18, Eurospher (250 X 4.6) mm, 5 µm Mobile phase: 50% acetonitrile + 50% aqueous solution (potassium phosphate dihydrogen 0.02 M, pH=5) v/ v Flow rate: 1 ml / min.

The result: only two peaks appeared.

- Variety ratios of the mobile phase were employed: three peaks appear two of them are overlapped.
- C8 column was used instead of the C18 using large amount of aqueous phase (acetonitrile: water (20: 80) v/v), with the same previous experimental conditions. Three separated peaks were obtained with good resolution and good symmetry. First peak is for metronidazole at retention time 6.118 minutes and the second is for Secnidazole at 8.193 minutes and the third is for Tinidazole at 9.977 minute. For confirmation, a solution of each compound was separately injected.

• In order to decrease the time of total analysis, the flow rate was increased to 1.5 ml/min. Retention time was decreased to 4.140 and 5.515 and 6.692 minutes, respectively.

The system suitability parameters were good and compatible with USP⁵ (Table 1).

Figure 3 shows the Chromatogram of the mixture of the three compounds.

2.6. Verification of the method's validity

Table 2 summarizes the results of the method validation results of the three compounds

2.7. Samples test results

• Raw materials

Table 3 shows the results of analysis of the raw materials of metronidazole, Tinidazole and Secnidazole.

Pharmaceutical Preparations

• Results of tablets samples of Metronidazole

Table 4 shows the results of analysis of the tablet samples of metronidazole. The percentage of active substance in each sample was calculated from the peaks areas of samples and standard solutions.

• Results of tablets samples of secnidazole

Table 5 shows the results of analysis of the tablet samples of secnidazole.

2.8. Serum samples

Standard serum solutions series were first injected using the mentioned Chromatographic conditions. Linearity was good in the range of (1 - 30) µg/ml. Peak areas of the standard serum series were compared with peak areas of standard series solutions and the recoveries were calculated (table 6). Figure 4 shows the chromatogram of a serum sample containing a mixture of 5 µg/ml of metronidazole, secnidazole and tinidazole.

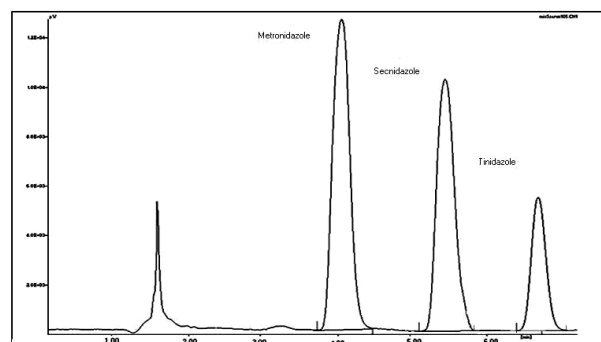


Figure 4: Chromatogram of a Serum Sample Containing a Mixture of 5µg /ml of metronidazole, Secnidazole and Tinidazole

Table 1: System suitability parameters

Compound	Retention time	Symmetry	Theoretical plates	Resolution
Metronidazole	4.140	1.283	7021	-
Secnidazole	5.515	1.278	7781	5.266
Tinidazole	6.692	1.268	7121	3.706

Table 2: Method Validation Results

Compound	Retention Time (min)	Linearity	Recovery %						Quantitation Limit µg/ml	Detection Limit µg/ml	
			Accuracy	Precision		Selectivity	Robustness				
				Repeatability	Intermediate		Flow rate ml/min				
Metronidazole	4.10	0.977	99.22	98.44 RSD=1.12	98.379 RSD=1.35	99.59	99.38	101.96	102.16	0.024	0.080
Secnidazole	5.532	0.998	99.89	101.08 RSD=1.27	100.03 RSD=0.99	100.93	101.01	101.25	101.47	0.049	0.164
Tinidazole	6.637	0.997	99.64	98.98 RSD=1.47	98.52 RSD=1.16	101.09	100.50	99.47	99.58	0.0748	0.249

Table 3: Raw Materials Sample Results

Factory	Compound	Average of Standard Peak Area*	Average of sample Peak Area**	Percentage of the Active Substance%
H1	Metronidazole	4386045	4341875	98.99
F1	Secnidazole	3626939	3649766	100.62
G1	Tinidazole	2947955	2876535	97.57

* Average of five injections of the standard; ** Average of three injections of the sample

Table 4: Results of tablet Samples of metronidazole

Factory	Tablet sample	Average of Standard Peak Area*	Average of sample Peak Area**	Percentage of the Active Substance%
A1	500 mg metronidazole	4196576	4137736	98.59
B1	500 mg metronidazole	4196576	4312088	102.75
D1	250 mg metronidazole	4025876	3981386	98.89

* Average of five injections of the standard; ** Average of three injections of the sample



Table 5: Results of tablet Samples of secnidazole

Factory	Tablet sample	Average of Standard Peak Area*	Average of sample Peak Area**	Percentage of the Active Substance %
C1	500 mg secnidazole	3612884	3555727	98.4
E1	500 mg secnidazole	3450582	3526171	102.19

* Average of five injections of the standard; ** Average of three injections of the sample

Table 6: Recovery Results of the Serum Samples

Number of sample	Concentration $\mu\text{g/ml}$	Recovery %		
		Metronidazole	Secnidazole	Tinidazole
1	1	88.40	96.96	90.78
2	5	91.74	92.51	89.69
3	10	91.80	91.18	94.92
4	20	90.25	88.71	91.62
5	30	88.66	89.07	89.09

3. DISCUSSION AND CONCLUSION

- A new, valid, sensitive, accurate and rapid analytical method has been developed in this study for the assay and separation mixture of metronidazole, Tinidazole and Secnidazole using column C8 mobile phase acetonitrile: water (80:20) v / v, UV detector at 318 nm and flow rate 1.5 ml / min.
- Pharmacopoeias (BP 2009⁷, E.P 2007⁸) had not yet mentioned any HPLC method for the assay of any of the studied drugs.
- Increasing water ratio in mobile phase from 50% to 80% led to good separation of the three compound.
- Increase flow rate from 1 to 1.5 ml/min reduced the retention times of metronidazole, secnidazole and tinidazole from 6.118, 8.193 and 9.977 to 4.140, 5.515 and 6.692 minutes, respectively.
- The percentage concentration of metronidazole and tinidazole obtained for the tested raw materials and tablets were in the USP range 99-101% for metronidazole and 98-101% for tinidazole.
- The tested tablets sample of metronidazole were also in the USP range 90-110%.
- The recovery percentages of spiked serum samples for metronidazole, secnidazole and tinidazole were 88 - 96%.
- This analytical method seem to be a good one for the determination of three compounds in raw materials as well as in tablets .It could be also used for the determination of their concentration in human serum in the range 1-30 $\mu\text{g/ml}$.

REFERENCES

1. Laurence L, Brunton, John S Lazo, Goodman & Gilman's The Pharmacological Basis of Therapeutics - 11th Ed, The Mcgraw-Hill Companies, Chapter 40, 2006.
2. Oreste A Mascaretti, Bacteria Versus Antibacterial Agents, ASM Press Us, Chapter 24 (2003)311.
3. Cristina Mahugo-Santana, Zoraida Sosa-Ferrera, Analytical Methodologies for the Determination of Nitroimidazole Residues in Biological and Environmental Liquid Samples, Analytica Chimica Acta 665 (2010)113-114.
4. H.Richard. Adams, Veterinary Pharmacology and Therapeutics, 8 edition, Iowa State university Press (2001) 992-1016.
5. USP Pharmacopoeia, 30/2007, 32 /2009 Editions.
6. Anailien Boza Riveraa, Rolando González Hernández, physico-Chemical and Solid-State Characterization of Secnidazole, J Il Farmaco 55 (2000) 700-707.
7. British Pharmacopoeia 2009, 2007 Edition.
8. European Pharmacopoeia 6.0 Edition 2007.
9. Bartlett Pn, Ghoneim E, El Hefnawy G, El-Hallag I. Voltammetry And Determination Of Metronidazole at a carbon fiber microdisk electrode, J.Talanta 66(4) (2005) 869-874.
10. Olajire Aremu Adegoke, A New Approach to the Spectrophotometric Determination of Metronidazole and Tinidazole Using P-Dimethylaminobenzaldehyde, Journal.acta pharmaceutica. 59(4) (2009) 407-419.
11. T. Saffaj, M. Charrouf, A., Spectrophotometric Determination of Metronidazole and Secnidazole In Pharmaceutical Preparations Based on the Formation Of Dyes, J.Dyes And Pigments, Volume 70, Issue 3 (2006) 259-262.
12. M Rajput, S Sinh, cofriendly Spectrophotometric Estimation Of Tinidazole In Tablets Using Lignocaine Hydrochloride As A Hydrotropic Solubilizing Agent, Asian Journal of Pharmaceutics, Volume 3 , Issue 4 (2009) 319-321.



13. Abdel Fattah M. El Waliy, Spectrophotometric And HPLC Determination of Secnidazole In Pharmaceutical Tablets, *Journal of Pharmaceutical And Biomedical Analysis* Volume 22, Issue 6 (2000) 887-897.
14. Safwan Ashour, Nuha Kattan, Simultaneous Determination Of Miconazole Nitrate And Metronidazole In Different Pharmaceutical Dosage Forms By Gas Chromatography And Flame Ionization Detector (GC-FID), *International Journal of Biomedical Science*, Vol. 6 , No. 1 (2010) 13-18.
15. Xiaoyu Li, Jianguo Sun, Determination of Secnidazole In Human Plasma By High-Performance Liquid Chromatography With UV Detection And Its Application To The Bioequivalence Studies, *Biomedical Chromatography*, volume 21, Issue 3 (2007) 304–309.
16. Qin Y, Zou Y, Et al, Determination of Metronidazole In Serum By HPLC, *Journal Of West China University Of Medical Sciences*, 27(1) (1996) 114-116.
17. Salvesen B, Leinebo O, Bergan T, Assay of Metronidazole By HPLC Compared With Microbial Method, *Scand J Gastroenterol Suppl* (1984) 31-43.
18. Krishnaiah Ysr, Estimation of Tinidazole In Pharmaceutical Dosage Forms By Reverse Phase HPLC, *Antiseptic*. 99(1) (2002) 5-7.
19. K. Rajnarayana, M. R. Chaluvadi, Validated HPLC Method For The Determination Of Tinidazole In Human Serum And Its Application in A Clinical Pharmacokinetic Study, *pharmazie* , Vol. 57 (2002) 535-537.
20. Jaber Emami, A Rapid And Sensitive Hplc Method For The Analysis Of Metronidazole In Human Plasma: Application To Single Dose Pharmacokinetic And Bioequivalence Studies, *Daru Journal Of Pharmaceutical Sciences* Volume 14, No. 1 (2006) 15-21.
21. Li Qun Ouyang, Hai Long Wu, Simultaneous Determination Of Metronidazole And Tinidazole In Plasma By Using HPLC-DAD Coupled With Second-Order Calibration, *Chinese Chemical Letters* 21 (2010) 1223–1226 .
22. Nilsson-Ehle, B Ursing, and P Nilsson-Ehle, Liquid chromatographic assay for metronidazole and tinidazole: pharmacokinetic and metabolic studies in human subjects, *J.Antimicrob Agents Chemother*. vol 19(5) (1981) 754–760.
23. M. A. Brooks, L. D'Arconte Determination of nitroimidazoles in biological fluids by differential pulse polarography, *Journal of Pharmaceutical Sciences*, Volume 65, Issue 1 (1976) 112–114.
24. Sun Hw, Wang Fc, Ai Lf., Simultaneous Determination of Seven Nitroimidazole Residues In Meat By Using Hplc-Uv Detection With Solid-Phase Extraction, *J Chromatogr B Analyt Technol Biomed Life Sci*. 857(2)(2007) 296-300.
25. Capitan-Vallvey F.Ariza, Liquid Chromatography-Mass Spectrometry Determination of Six 5-Nitroimidazoles In Animal Feedstuff, *Chromatographia*, Vol. 65 (2007) 283-290.
26. Mark Cronly, Patrice Behan, Barry Foley, Edward Malone, Liam Regan, Rapid confirmatory method for the determination of 11 nitroimidazoles in egg using liquid chromatography tandem mass spectrometry, *Journal of Chromatography A*, Publisher: Elsevier B.V., Volume 1216, Issue 46 (2009) 8101-8109.
27. Patel Y.; Dhorda U.J.; Sundaresan M.1; Bhagwat A.M., Separation And Estimation Of Five Imidazoles By Packed Column Supercritical Fluid Chromatography, *analytica Chimica Acta*, Volume 362 (2) (1998) 271-277.
28. Clare Ho, Della W.M. Sin, Determination of dimetridazole and metronidazole in poultry and porcine tissues by gas chromatography–electron capture negative ionization mass spectrometry, *Analytica Chimica Acta*, Volume 530, Issue 1(2005)23-31.
29. J Polzer, P Gowik, Validation of a method for the detection and confirmation of nitroimidazoles and corresponding hydroxy metabolites in turkey and swine muscle by means of gas chromatography-negative ion chemical ionization mass spectrometry, *J Chromatogr B Biomed Sci Appl*. 761(1) (2001) 47-60.
30. Savfaraz K. Niazi, *Handbook Of Pharmaceutical Formulations* Crc Press Llc , Volume 1 (2004) 174-177.
31. Anthony C Moffat, M David Osseelton, Brian Widdop, Clarke`S *Analysis of Drugs and Poisons*, Third Edition 2004.

