IJPSR (2013), Vol. 4, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 01 March, 2013; received in revised form, 21 May, 2013; accepted, 29 June, 2013

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE DETERMINATION OF METRONIDAZOLE BENZOATE AND RELATED IMPURITES IN BULK AND PHARMACEUTICAL FORMULATIONS

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Keywords:

HPLC, Validation, Metronidazole benzoate, Related impurities, Metronidazole, 2-methyl-5-nitromidazole, Benzoic acid

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ABSTRACT: A simple, selective, precise and reliable HPLC method of analysis has been developed and validated for determination of the metronidazole benzoate and related impurities, including metronidazole, 2-methyl-5-nitromidazole and benzoic acid in bulk and marketed formulations. The method employed CN-RP column as stationary phase and acetonitrile and 0.1% octansulfonic acid sodium salt as mobile phase. The method was validated in terms of linearity, precision and accuracy. The LOD and LOQ were 0.076, 0.031, 0.033 and 0.024 µg/ml and 0.232, 0.095, 0.098 and 0.073 µg/ml respectively for metronidazole benzoate, metronidazole, 2-methyl-5-nitromidazole and benzoic acid. The average percentage recovery of metronidazole benzoate and related impurities was found to be within 98.6 - 101.5 % of range. The developed method can be successfully used for identification and quantification of metronidazole benzoate and related impurities in bulk and formulations.

INTRODUCTION: Metronidazole is an antimicrobial agent that has been used in clinical medicine for >45 years [1]. It was originally indicated for the management of infection caused by *Trichomonas vaginalis* and was then shown to be effective against other protozoal infections, such as amebiasis and giardiasis. Metronidazole is highly active against gram-negative anaerobic bacteria, such as *Bacteroides* and gram-positive anaerobic bacteria, such as *Clostridium difficile.* Treatment regimens for the eradication of *Helicobacter pylori* still include metronidazole in combination with other agents.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.4(7).2594-99

Article can be accessed online on: www.ijpsr.com

Metronidazole is also indicated for the treatment of bacterial vaginosis caused by *Gardnerella vaginalis.* Despite 45 years of extensive use, metronidazole remains the criterion standard for the management and prophylaxis of anaerobic infections. The pharmacokinetic and pharmacodynamic properties of the drug are favorable, and it is available as oral, intravenous, vaginal, and topical formulations [2-4].

The mechanism of metronidazole and other nitro imidazoles action have not yet been fully elucidated but include the inhibition of DNA synthesis and DNA damage by oxidation, causing single-strand and double-strand breaks that lead to DNA degradation and bacterial cell death. The metronidazole molecule is converted to a short-lived nitroso free radical by intracellular reduction, which includes the transfer of an electron to the nitro group and yielding cytotoxic form of the drug that can interact with the DNA molecule [5, 6].

Aerobic cells lack electron transport proteins with sufficient negative redox potential and therefore, the drug is active against only bacteria with anaerobic metabolisms and some microaerophils, such as *H. pylori*. In anaerobic bacteria, the electron acceptors including flavodoxin and ferredoxin, (which receive electrons from the pyruvate-ferredoxin oxireductase complex), have a reduction potential lower than that of the metronidazole molecule and will thereby donate its electrons to the drug. In addition, reoxidation can occur in the presence of molecular oxygen and can convert the compound back to its original inactive form [7].

The safety profile of metronidazole is well known, and adverse effects are considered mainly to be mild to moderate in severity. The most common adverse reactions reported involve the gastrointestinal tract. Rare serious adverse reactions, including convulsive seizures and peripheral neuropathy, characterized mainly by numbness or paresthesia of an extremity, have been reported in patients receiving prolonged metronidazole treatment [8].

Impurity profiling (ie, the identity as well as the quantity of impurity in the pharmaceuticals), is now receiving important critical attention from regulatory authorities. Impurities in metronidazole benzoate pharmaceutical formulations including metronidazole and benzoic acid are the unwanted chemicals that remain with the active ingredients or during develop formulation, or upon aging of both metronidazole benzoate bulk and formulated medicines. The presence of these unwanted chemicals even in small amounts may influence the eff8ticacy and safety of the pharmaceutical products [9].

The different pharmacopoeias, such as the BP and USP, are slowly incorporating limits to allowable levels of impurities present in the APIs or formulations. Moreover, literature survey reveals that, metronidazole benzoate impurities are no official in any of the pharmacopeias like IP, BP, USP and EP. Hence an attempt has been made to develop a simple, efficient and selective method for the determination of metronidazole benzoate and related impurities, including 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethanol (metronidazole), 2-methyl-5-nitromidazole and benzoic acid in bulk and pharmaceutical dosage forms (**Figure 1**).

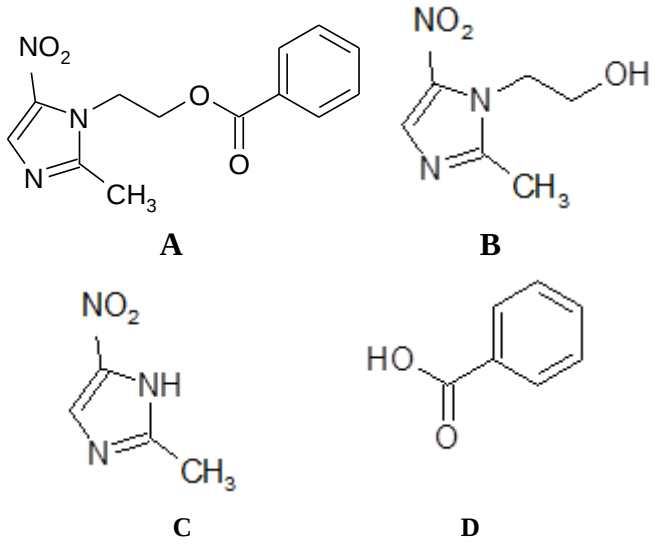

FIGURE 1: STRUCTURE OF METRONIDAZOLE BENZOATE (**A**), 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethanol (metronidazole - **B**), 2-methyl-5-nitromidazole (**C**) and benzoic acid (**D**).

MATERIALS AND METHODS

Instrumentation: A High Performance Liquid Chromatography (HPLC) method of metronidazole benzoate and related impurities analytical method was developed on LCMS-PDA-M20A Shimadzu with diode array detector. EC NUCLEOSIL CN-RP (250x4, 6mm, 5µ packing, Machery-Nagel, Germany) column was used. The elution was carried out isocratically.

Chemicals: Ocatansulfonic acid sodium salt (Sigma-Aldrich, purity 98%, batch:117K0099), Millipore water, acetornitrile (HPLC grade, Alpha chemika; purity: 99.9%, batch: A5982;), metronidazole benzoate CRS; (E P Reference Standard; purity: 100%, code: M1851000; batch: 1,2; 002TV3), 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (KRKA; purity: 99,8%, batch: 368459), 2-methyl-5-nitromidazole (KRKA; purity: 98,9%, batch: 0885V) and benzoic acid (Acros Organics; purity: 99,9%; batch: A0250626) were used in this study.

Preparation of stock solution and working standard solution:

1. **Preparation of Mobile Phase:** 1.0 gm of ocatansulfonic acid sodium salt was weighed and transferred into a 1000 ml beaker, dissolved and diluted with 1000 ml water. 800 ml of 0.1% octansulfonic acid sodium salt and 200 ml acetonitrile were mixed.

The mobile phase was filtered through 0.45µm membrane filter under vacuum filtration and was degassed before used, then delivered at a flow rate 1.0 ml/min.

2. **Preparation of standard solution:** 5.0 mg of metronidazole benzoate reference standard was weighed and transferred into 25 ml volumetric flask. 10 ml of mobile phase was added, sonicated for 5 min, mixed thoroughly to dissolve and make up the volume to 25 ml with mobile phase.

3. **Preparation of sample solution:** 5.0 mg of metronidazole benzoate active substance was weighed and transferred into 25 ml volumetric flask. 10 ml of mobile phase was added, sonicated for 5min, mixed thoroughly to dissolve and make up the volume to 25ml with mobile phase. Sample solution was filtered through 0.45µm membrane filter.

Quantification Limits: The quantification limit was defined as the lowest fortification level evaluated at which acceptable average recoveries were achieved and analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of detection (LOD) and quantification (LOQ) was defined as LOD=3.3 S/K and LOQ=10 S/K respectively**.** Where 'S' is the standard deviation of replicate determination values; 'K' is the sensitivity namely the slope of the calibration graph.

Calibration curve: The calibration curve was constructed by plotting peak area concentration of metronidazole benzoate and impurities standard solutions. Aliquots of standard stock solutions of metronidazole benzoate and impurities in the concentration range 1-100 µg/ml was transferred into 25 ml volumetric flask and 10 ml of mobile phase was added, Sonicated for 5 min, mixed thoroughly to dissolve and make up the volume to 25 ml with mobile phase. Each concentration 20µl of the standard solutions was injected and the chromatograms were recorded. The calibration graph was done by external standard calibration method.

Accuracy: Accuracy was determined for standard quality samples (in addition to calibration standard) prepared in triplicates at different concentration levels (5, 50, 100 µg/ml.) within the range of linearity of metronidazole benzoate and impurities. The results of analysis of recovery studies were obtained by method validation by statistical evaluation.

Precision: The precision of the instruments was checked by repeatedly (intraday) intermediate (inter day) and reported as % RSD for a statistically significant number of replicate measurements. Repeatability and intermediate precision of the method were determined by analyzing 5 samples of the test concentration 0.05 mg/ml.

Specificity: The specificity of the assay method is established by injecting blank, standard, excipients mixture and sample of metronidazole benzoate into the HPLC. The identity of metronidazole benzoate was confirmed by comparison of its retention time (R_T) and UV-spectra.

Quantification of formulation (Assay): 5.0 mg of metronidazole benzoate reference standard was weighed and transferred into 25 ml volumetric flask. 10 ml of mobile phase was added, sonicated for 5 min, mixed thoroughly to dissolve and make up the volume to 25 ml mobile phase.

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Sample solution was filtered through 0.45 µm membrane filter. 20 µl of each solution was injected and chromatograms were recorded. The peak area was determined and the procedure was repeated for six times. By using the following formula the percentage purity of metronidazole benzoate was calculated.

$$\frac{AT}{AS} x \frac{WS}{DS} x \frac{DT}{WT} x \frac{P}{100} x 100\%$$

Where: AT = Peak area of metronidazole benzoate obtained with test preparation; AS = Peak area of metronidazole benzoate obtained with standard preparation; WS = Weight of working standard in mg; WT = Weight of working sample taken in mg; DS = Dilution of standard solution; DT = Dilution of sample solution and P = Percentage purity of working standard.

RESULTS AND DISCUSSION: The HPLC method for metronidazole benzoate and related impurities, including 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethanol (metronidazole), 2-methyl-5-nitromidazole and benzoic acid was optimized (**Table 1**). The mobile phase acetornitrile: 0.1% octansulfonic acid sodium salt (20:80 v/v) gave sharp and well defined peaks with significant R_T values which were desired for quantification of metronidazole benzoate and related impurities in bulk and formulations (**Table 2**). The method showed specificity because metronidazole benzoate and related impurities, including metronidazole, 2-methyl-5-nitromidazole and benzoic acid were well-resolved and no interfering peaks were observed as it appears in **Figure 2**. Retention times were 2, 05 min for benzoic acid, 3,71 min for 2-methyl-5-nitromidazole, 4,2 min for metronidazole and 8,4 min for metronidazole benzoate.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES.

Parameter/Condition	Specification
Column	EC NUCLEOSIL CN-RP (250x4,6mm, 5µ packing)
Mobile phase	Acetornitrile:0.1% octansulfonic acid sodium salt (20:80 v/v)
Working wavelength	235 nm
Flow rate	1 ml/min
Column temperature	40^{o}C
Sample volume	20 uL
Run time	15 min

TABLE 2: SPECIFICITY OF HPLC METHOD FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES*

Parameters	metronidazole benzoate	metronidazole	2-methyl-5-nitromidazole	benzoic acid
R_T	8,426 ± 0,096	4,205 ± 0,002	3,71 ± 0,002	2,0525 ± 0,004
Peak area	7870372,2 ± 176002,6	2322744 ± 1627,3	3710625± 8575,1	5878006,7±22177,5
R_T %RSD¶	1,139	0,065	0,058	0,209
Peak area RSD¶	2,236	0,070	0,231	0,377

*All data represent Mean ± SD for n=6 standard samples for each of mentioned analyte. Grubbs test detects no outliers from normal distribution (α = 0.02). ¶ %RSD = 100 × (SD/Mean)

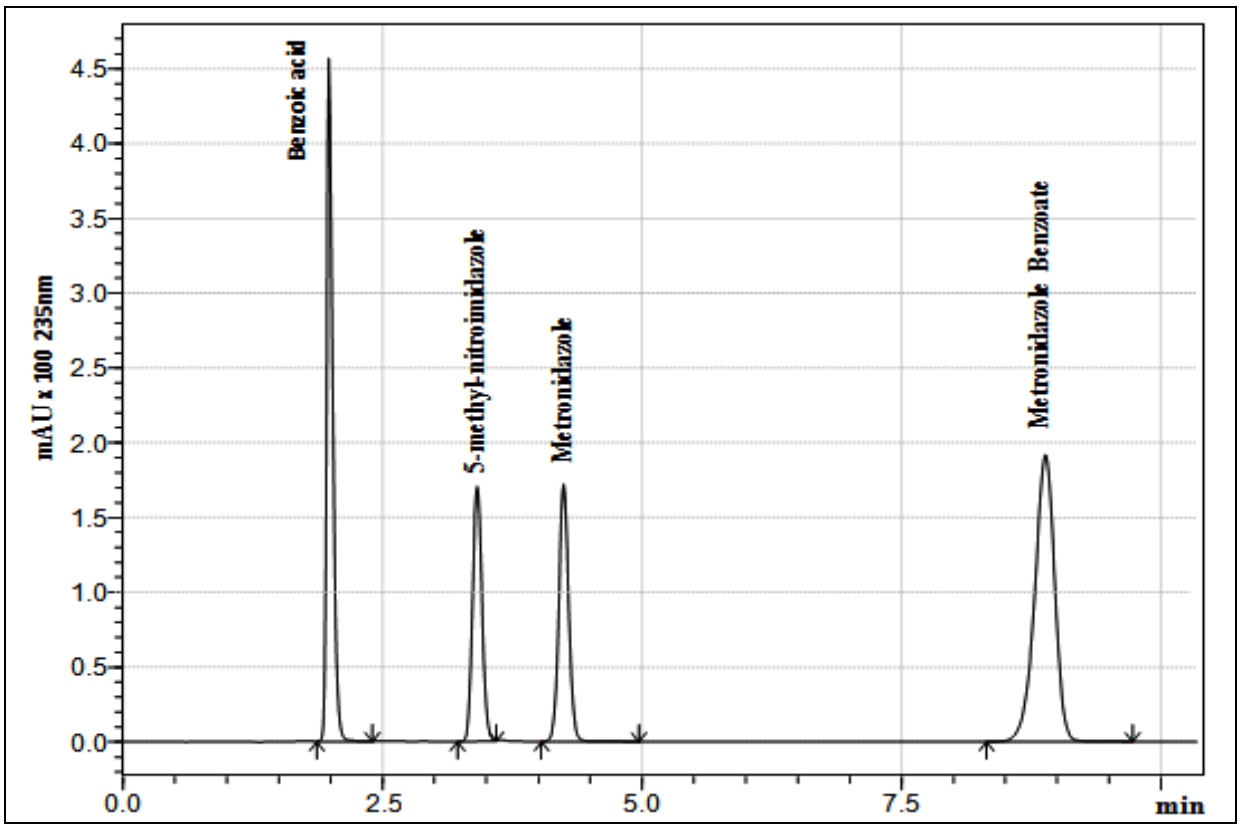

FIGURE 2: OPTIMIZED HPLC CHROMATOGRAM FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES

TABLE 3: REGRESSION ANALYSIS OF THE CALIBRATION CURVE FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES FOR THE PROPOSED HPLC METHOD

Parameters	Metronidazole benzoate	Metronidazole	2-methyl-5-nitromidazole	Benzoic acid
Concentration range	1-100 µg/ml	1-100 µg/ml	1-100 µg/ml	1-100 µg/ml
Slope	2.668	8.429	5.297	3.069
Intercept	0.090	0.122	0.308	0.536
Correlation coefficient	0.9998	0.9998	0.9995	0.9996
Regression equation	Y=2.66X - 0.09	Y=8.429X - 0.122	Y=5.29X + 0.308	Y=3.069X -0.53
LOD (µg/ml)	0.076	0.031	0.033	0.024
LOQ (µg/ml)	0.232	0.095	0.098	0.073

The calibration curves in this study were plotted between amount of each of analyte versus peak area and the regression equations with a regression coefficient were obtained. The linear regression data (**Table 3**) showed good linear relationship over a concentration range of 1-100 µg/ml for metronidazole benzoate, metronidazole, 2-methyl-5-nitromidazole and benzoic acid. Regression equation for metronidazole benzoate was Y=2.66X - 0.09 with a regression coefficient of 0.9998, and Y=8.429X - 0.122 with a regression coefficient of 0.9998 for metronidazole, and Y=5.29X + 0.308 with a regression coefficient of 0.9995 for 2-methyl-5-nitromidazole and Y=3.069X -0.53 with a regression coefficient of 0.9996 for benzoic acid.

In order to determine the detection and quantification limit, analytes concentration in the lower part of calibration curve was used. Metronidazole benzoate and related impurities solutions of 1µg/ml were prepared and analyzed using five replicates and the amount of each analyte peak area was determined. The LOD and LOQ values for metronidazole benzoate and related impurities are shown in **Table 3**.

The intraday precision was determined by measurement of analyte concentration using five replicates of metronidazole benzoate and related impurities 50 µg/ml solutions two times on the same day and inter day variations were determined similarly on consecutive days. The repeatability of sample application was assessed five times on HPLC followed by recording of the amount of metronidazole benzoate and related impurities 50 µg/ml sample solutions. The % RSD for peak values of metronidazole benzoate was found to be 1.113% and 1.016% for intra and inter-day precision respectively. The % RSD and results for related impurities including metronidazole, 2-methyl-5-nitromidazole and benzoic acid are depicted in **Table 4**, which reveal intra and inter day variations of analytes concentration.

TABLE 4: INTRADAY AND INTER DAY PRECISION OF HPLC METHOD FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES 50 µg/ml SOLUTIONS

Parameters	Metronidazole benzoate	Metronidazole	2-methyl-5-nitromidazole	Benzoic acid
Concentration*	51.19	50.058	49.775	51.92
SD	0.57	1.878	1.762	0.335
Intraday %RSD ¶	1.113	3.752	3.54	0.645
Inter day %RSD ¶	1.016	4.688	2.064	2.091

*Mean and SD represent for n=5 standard samples for each of mentioned analyte. ¶ %RSD = 100 × (SD/Mean).

TABLE 5: RECOVERY ANALYSIS OF THE HPLC METHOD FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES

Parameters	Metronidazole benzoate	Metronidazole	2-methyl-5-nitromidazole	Benzoic acid
Amount of analyte added (µg/ml)	5	5	5	5
	50	50	50	50
	100	100	100	100
Amount of analyte found (µg/ml) *	5.03 ± 0.24	5.05 ± 0.18	5.24 ± 0.10	4.66 ± 0.87
	51.19 ± 0.57	50.06± 1.88	49.77 ± 1.76	51.92 ± 0.34
	96.37 ± 4.32	101.43 ± 1.02	100.34 ± 2.02	98.89 ± 0.41
Recovery %	100.6	101.0	104.8	93.2
	102.4	100.1	99.5	103.8
	96.4	101.4	100.3	98.9
Average %	99.8	100.8	101.5	98.6

*Mean and SD represent for n=5 standard samples for each of mentioned analyte.

Recovery studies of the samples were carried out for the accuracy parameter. These studies were carried out at three levels; sample solutions of 5, 50 and 100 µg/ml were prepared and recovery studies were performed using five replicates. Percentage recovery was found to be within the limits as listed in **Table 5**.

The "Metronidazole Denta, gel for gums" formulation, produced by Arpimed LLC (Abovyan, Armenia) was selected for analysis and procedure was repeated for three times by validated HPLC method. The percentage of API metronidazole benzoate purity was found to be 97.4 % and related impurities 0.016 % for metronidazole and 0.055 % for benzoic acid and not 2-methyl-5-nitromidazole were found by validated HPLC method (**Table 6**). The RSD % was found to be less than 1% which indicates that the method had good precision.

TABLE 6: ANALYSIS OF "METRONIDAZOLE DENTA, GEL FOR GUMS" FORMULATION BY THE PROPOSED HPLC METHOD FOR METRONIDAZOLE BENZOATE AND RELATED IMPURITIES.

Parameters	Metronidazole benzoate	Metronidazole	2-methyl-5-nitromidazole	Benzoic acid
% Required*	92.5-107.5	≤ 0.5	≤ 0.1	≤ 0.5
% Found	97.41±0.03	0.0169±0.0001	not found	0.0554±0.0001
%RSD ¶	0.03	0. 59	-	0.18

*Mean and SD represent for n=3 standards and samples. ¶ %RSD = 100 × (SD/Mean).

A new, accurate and selective HPLC method were proposed for the determination of metronidazole benzoate and related impurities, including metronidazole, 2-methyl-5-nitromidazole and benzoic acid in bulk and in dosage forms validated as per the ICH guidelines. The methods were found to be simple, selective, precise and accurate. Therefore, these methods can be used as routine testing as well as stability analysis of metronidazole benzoate in bulk and in formulations. All statistical results (Percentage, Mean, RSD, Percentage difference and recovery %) were within the acceptance criteria.

CONCLUSION: A new, accurate and selective HPLC method were proposed for the determination of metronidazole benzoate and related impurities, including metronidazole, 2-methyl-5-nitromidazole and benzoic acid in bulk and in dosage forms validated as per the ICH guidelines. The methods were found to be simple, selective, precise and accurate. Therefore, these methods can be used as routine testing as well as stability analysis of metronidazole benzoate in bulk and in formulations. All statistical results (Percentage, Mean, RSD, Percentage difference and recovery %) were within the acceptance criteria.

AKNOWLEDGEMENTS: We thank General Manager of Arpimed LLC, Dr. Vachagan Ghazaryan, for his support of this study.

REFERENCES:

1. Löfmark S., Edlund C., Nord C.E. Metronidazole is still the drug of choice for treatment of anaerobic infections, Clinical Infectious Diseases, 50, 2010, S16–23.
2. Nassour I., Carchman E.H., Simmons R.L., Zuckerbraun B.S. Novel management strategies in the treatment of severe Clostridium difficile infection, Advances in Surgery, 46, 2012, 111-135.
3. Stover K.R., Riche D.M., Gandy C.L., Henderson H. What would we do without metronidazole? American Journal of the Medical Sciences, 343, 2012, 316-319.
4. Chuah S.K., Tsay F.W., Hsu P.I., Wu D.C. A new look at anti-Helicobacter pylori therapy, World Journal of Gastroenterology, 17, 2011, 3971-3975.
5. Soares G.M., Figueiredo L.C., Faveri M., Cortelli S.C., Duarte P.M., Feres M. Mechanisms of action of systemic antibiotics used in periodontal treatment and mechanisms of bacterial resistance to these drug, Journal of Applied Oral Science, 20, 2012, 295-309
6. Land K.M, Johnson P.J. Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa, Drug Resistance Updates, 2, 1999, 289–294.
7. Diniz C.G., Santos S.G., Pestana A.C., Farias L.M., Carvalho M.A., Auxiliadora M. Chromosomal breakage in the B. fragilis group induced by metronidazole treatment, Anaerobe, 6, 2000, 149–153.
8. Kuriyama A., Jackson J.L., Doi A., Kamiya T.Metronidazole-induced central nervous system toxicity: a systematic review, Clinical Neuropharmacology, 34, 2011, 241-247.
9. Rao N.R., Kiran S.S., Prasanthi N.L. Pharmaceutical Impurities: An overview, Indian Journal of Pharmaceutical Education and Research 44, 2010, 301-310.

How to cite this article:
Melikyan LA, Martirosyan SS, Grigoryan RS, Topchyan HV and Davtyan TK: Development and validation of HPLC method for the determination of Metronidazole benzoate and related impurities in bulk and pharmaceutical formulations. *Int J Pharm Sci Res* 2013: 4(7); 2594-2599. doi: 10.13040/IJPSR. 0975-8232.4(7).2594-99