6.1. DRUG PROFILE

6.1.1. Nitrofurantoin

Nitrofurantoin (NIT) [1, 2] is bactericidal agent and its major purpose is to treat urinary tract infections [3-7]. It is also active against some gram positive bacteria and some gram negative bacteria. NIT exhibits complex and unique mechanism. In particular, the reduced form of NIT is highly reactive and selectively damages the DNA [8] of bacteria. Thus, it effects the growth of the bacteria rather than killing the bacteria. Due to extremely poor tissue penetration, NIT is not suggested for the treatment of pyelonephritis [9], intra-abdominal abscess [10, 11] and prostatitis [12]. It is also not recommended for people who are suffering from kidney problems. Headache, diarrhoea, loss of appetite and nausea are the certain common side effects. Lung problems, liver problems or numbness may occur rarely.

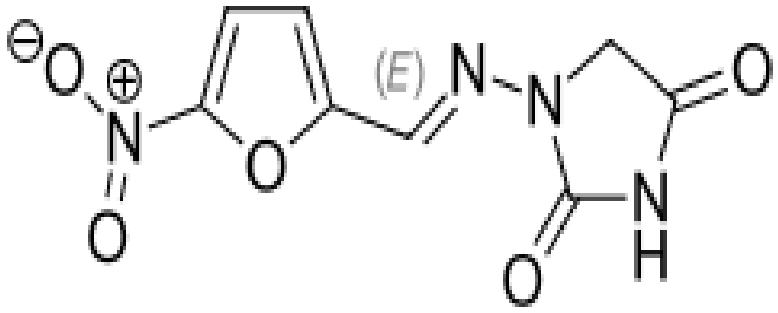


Figure.6.1. Structure of nitrofurantoin

IUPAC name: 1-[(E)-[(5-nitrofuran-2-yl) methylidene]amino]imidazolidine-2,4-dione

Molecular formula: $C_8H_6N_4O_5$

Molecular weight: 238.2

Melting point: 272^0C

Solubility: Water

Brand name: Furadonine, Macrodantin and Furadantin.

6.1.2. Phenazopyridine

Phenazopyridine (PHE) [13] is an anesthetic generally used as local anesthetic in the treatment of urinary tract disorders [14-16]. PHE is considered as the derivative of amino pyridines. It is also used to treat lower urinary tract irritation. USP pharmacopoeia adopted PHE in the year 1928 as official drug [17, 18]. The mechanism of action of PHE is not known with certainty. PHE causes a vivid colour change in urine and this effect is useful to identify the presence of PHE in the body. That is the reason why PHE users are warned not to use contact lenses, otherwise PHE exhibits a permanent discolouration of contact lenses or fabrics [19]. The regular symptoms observed in PHE users are headache and dizziness. Yellow colouration of nails [20, 21] is noticed when the drug is used for a long period of time. Fever, confusion, swelling of face, fingers, feet or legs, skin rash and shortness of breath are the general side effects.

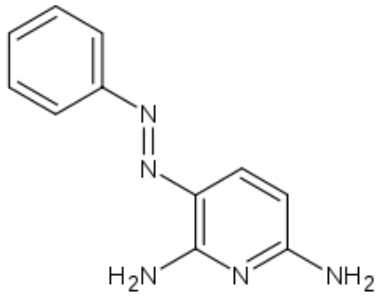


Figure.6.2. Structure of phenazopyridine

IUPAC name: 3-[(E)-2-phenyldiazen-1-yl] pyridine-2, 6-diamine

Molecular formula: $C_{11}H_{11}N_5$

Molecular weight: 213.2

Melting point: 139^0C

Solubility: Water

Brand name: Baridium, Pyridium and Uristat.

6.2. LITERATURE SURVEY

Numerous analytical methods were reported for the determination of NIT and PHE simultaneously in the pure form and in pharmaceutical formulations. Some colourimetric[22], spectrophotometric[23-30] and HPLC[31-39] methods were proposed for the estimation of NIT and PHE separately, in the combined form and in combination with other drugs. Plenty of analytical methods were described for the quantitative determination of NIT and PHE in biological fluids[40-65]. Yet, only fewer stability indicating methods[66] were suggested and hence the current investigation was greatly concentrated on stability indicating work.

Table.6.1: List of brand names of combined formulations of nitrofurantoin and phenazopyridine

S.No	Brand name	Available strength		Formulation	Manufacturer
1	Nephrogesic	Nitrofurantoin, Phenazopyridine	100mg 50mg	Tablet	Johnson & Johnson

Parth and Jignesh [24] described a simple and sensitive UV spectrophotometric absorption correction method for the estimation of PHE HCl and Ciprofloxacin hydrochloride (CIP HCl). Solvent utilized for the determination was methanol. Measurements were made at two wavelengths 279nm and 413nm for solving an equation. In case of PHE HCl, measurements were made at both the wavelengths but CIP HCI exhibited zero absorbance at 413nm and hence in case of CIP HCI measurements were made at 279nm only. Linearity was found to be good in the range of 2-10µg/ml for PHE HCl and was 2.5-12.5µg/ml for CIP HCI. Accuracy of the method was measured in terms of recovery. The mean recovery of PHE HCl was 95.56% and was 100.63% for CIP HCI. The proposed method was found to be accurate, reproducible, precise and economical and thus applicable for the simultaneous estimation of PHE HCl and CIP HCI in commercial formulation.

Sarfaraz and Reddy [30] illustrated a simple visibe spectrophotometric method for the development and validation of PHE in commercial formulation. At 422nm PHE showed maximum absorbance. Estimation of PHE was done by its reaction with diazonium salt of 4-aminobenzene sulphonic acid (PABSA). The reaction between PHE and PABSA resulted in the formation of red coloured azo derivative (λmax =508nm). Beer's law was obeyed in the concentration range of 1-15µg/ml. Optical, analytical and statistical parameters were determined and were found to be within the limits. Recovery of the solution, on the average was found to be 95.5%.

Pola and Gowri [31] proposed a simple, accurate and economical RP-HPLC method for the simultaneous estimation of ciprofloxacin (CIP) and PHE in pharmaceutical formulation. C18 column containing a mixture of ammonium orthophosphate and acetonitrile (50: 50, v/v) was utilized for the chromatographic separation at 275nm. The pH of the mobile phase was adjusted to 3.5 by dilute ammonia solution. Run time of the entire analysis was fixed at 8min. Validation parameters were represented in **Table.6.2.**

Table.6.2. Results of ciprofloxacin and phenazopyridine by RP-HPLC method

S.No	Parameter	Ciprofloxacin	Phenazopyridine
1	Retention Time	2.783min	4.11min
2	Linearity	25-150µg/ml	10-60µg/ml
3	%RSD of intra-day precision)	0.91	1.03
4	%RSD of inter-day precision)	0.79	0.83
5	% recovery	99.29 - 100.57	99.36 – 100.02
6	LOD	1.44µg/ml	0.964µg/ml
7	LOQ	4.38µg/ml	2.92µg/ml

Shetty [34] et al prescribed a simple and rapid RP-HPLC method for the quantitation of CIP and PHE. Analytical wavelength chosen was 295nm and the mobile phase selected was a mixture of methanol and water in 80: 20 ratios. Retention time exhibited by CIP was 4.91min and PHE was 2.85min. Linearity was established in the range of 5-25µg/ml for CIP and in the range of 10-50µg/ml for PHE. Different parameters were examined by making use of ICH guidelines. The percentage RSD of intra-day and inter-day precision was found to be within the acceptable range.

Zhang [37] et al were succeeded in developing a reliable, rapid and convenient HPLC method for the quantitative determination of NIT and furazolidone (FUZ) in cosmetics. Acetonitrile and methanol (1:1, v/v) was utilized for the extraction of several cosmetic products such as cream, water powder, lipstick, emollient and lotion under ultrasonication. At 25^0C, the separation was achieved on kromasil C18 column with acetonitrile and acetic acid (0.4%) in 30: 70 ratios as mobile phase. Diode array detector was used to perform the detection at 365nm. Linearity was established in the range of 0.1-20mg/l for both NIT and FUZ. Accuracy of the method was found to be in the range of 88.0% - 104.6%. Thus the proposed method was suitable for the estimation of NIT and FUZ in cosmetics.

Mandal and Ace [39] interpreted and validated RP-HPLC method for the analysis of NIT in rabbit plasma with acetanilide as internal standard at 270nm. The analysis was performed on C18 column by using a mixture of water and methanol (75: 25, v/v) as mobile phase at a flow rate of 9ml/min. The observed retention time for NIT was 58min and for that of acetanilide was 101min. Recovery of NIT was found to be higher than 92% when the concentration range of NIT was 5µg/ml. This method was applicable for the analysis of plasma samples of rabbits collected over time followed by the oral administration of NIT tablets.

Kai-jun [53] et al illustrated a simple, selective and sensitive GC-MS method for the determination of PHE in human plasma using diazepam as an internal standard (IS). DB-5MS column with mass selective detector was utilized for the analysis of PHE and IS which were extracted from plasma. Excellent linearity was found in the range of 5-500ng/ml for PHE. Relative standard deviation of intra-day and inter-day precision were found to be 1.37-6.69% and 1.24-6.01% respectively. Percentage recoveries were found to be in the range of 92.65-96.21. More than 90% extraction efficiency was obtained. Two formulations of PHE were examined in eighteen healthy male volunteers and successful results were obtained in terms of pharmacokinetics and bioequivalence.

Attia [66] et al demonstrated four simple, precise and accurate stability indicating spectrophotometric methods for the estimation of PHE HCl and 2,3,6-triaminopyridine (degradation product). Validation parameters were determined through all the four methods viz first derivative, derivative ratio, ratio difference spectrophotometric and dual wavelength method. On comparison of accuracy and precision results of the proposed methods with the reported method, it showed no significant difference.

6.3. EXPERIMENTAL

6.3.1. Chemicals and solvents

Pfizer pharmaceuticals gave the drug samples of nitrofurantoin and phenazopyridine as gift and the working standard of NIT and PHE was obtained as gift by Ranbaxy Laboratories Ltd. The pharmaceutical formulation was procured from provincial market. Methanol, acetonitrile and water of HPLC grade were purchased from Merck Specialties Private Limited, Mumbai, India. Buffer solutions of AR grade were purchased from Merck Specialties Private Limited, Mumbai, India.

6.3.2. Preparation of standard stock solution

Standard stock solution of NIT and PHE was prepared by the transferring accurately weighed 50mg of NIT and 100mg of PHE into 10ml volumetric flasks individually. Separately, NIT and PHE were dissolved accurately in 10ml methanol completely through sonication to obtain the respective standard stock solutions. These standard solutions were used for the preparation of corresponding working standard solutions i.e. 5-30µg/ml for NIT and 10-60µg/ml for PHE, through the method of dilution.

6.3.3. Preparation of sample solution

Twenty tablets of Nephrogesic brand (Nitrofurantoin 50mg and Phenazopyridine 100mg) were grounded to uniform size. An amount of 10mg of NIT was accurately weighted and was transferred into 10ml volumetric flask. Initially, 5ml of methanol was added into volumetric flask and NIT was dissolved in it. Complete dissolution and degasification were done through sonication (15min). Sufficient quantity of mobile phase was added to get the required volume. A concentration of 20µg/ml of NIT was obtained by the dilution with mobile phase. Simultaneously, a concentration of 40µg/ml of PHE was obtained as on the label claim. This combined solution was utilized for the simultaneous analysis of NIT and PHE in the combined dosage form.

6.3.4. Preparation of buffer solution

Sodium acetate and glacial acetic acid were used for the preparation of sodium acetate buffer solution. Initially, 2.99g of sodium acetate was weighed accurately and was dissolved completely in 500ml of HPLC water. At the same time, 1.66ml of glacial acetic acid was measured accurately and transferred into 498.34ml of HPLC water so as to obtain the volume of 500ml. These two solutions were mixed properly and completely. Thus 1000ml of sodium acetate buffer solution with a pH of 4.5 was obtained.

6.4. METHOD DEVELOPMENT

6.4.1. Detection of wavelength

At a wavelength of 248nm the two drugs NIT and PHE exhibited maximum overlapping and hence the entire analysis was carried out at this wavelength only.

6.4.2. Choice of stationary phase

Different octadecyl columns were examined in particular kromasil column, hypersil column and hypersil BDS C18 column. On hypersil BDS C18 column, NIT and PHE exhibited appropriate peak response. Consequently, hypersil BDS C18 column was utilized for the estimation of NIT and PHE.

6.4.3. Selection of mobile phase

Appropriate mobile phase for the analysis of NIT and PHE was selected on comparison of system suitability conditions in each and every trial. At last, mobile phase consisting of methanol and acetate buffer (75: 25, v/v) was found to be most suitable for the determination due to the display of good base line separation, high resolution and column efficiency.

6.4.4. Flow rate

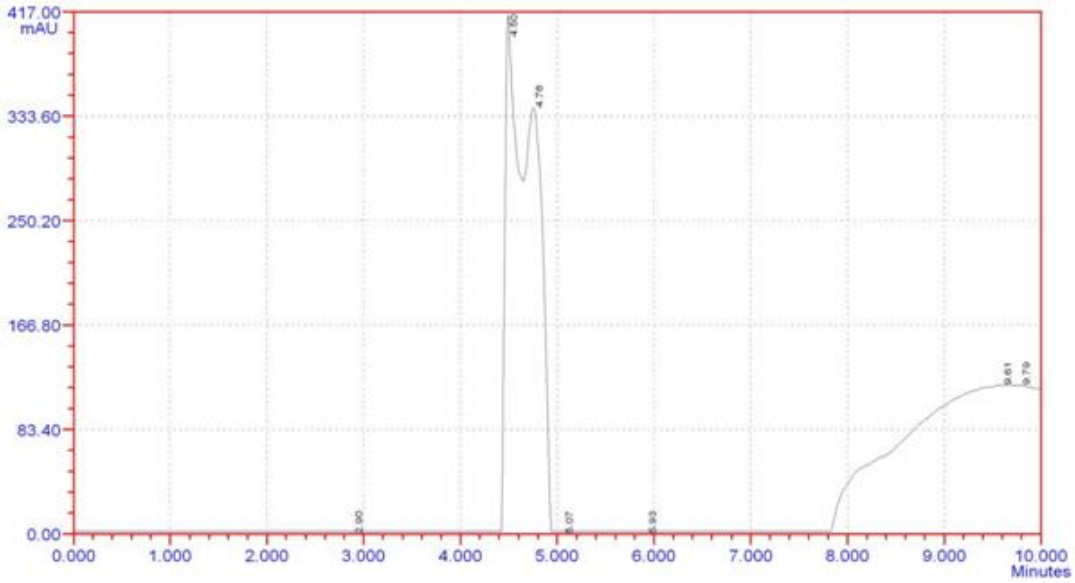
At a flow rate of 1.0ml/min NIT and PHE exhibited optimum separation.

6.4.5. Optimized chromatographic conditions

Numerous systematic trials (**Table.6.3**) were conducted to monitor optimized chromatographic conditions for the analysis of NIT and PHE. Trial chromatograms were shown from **Figure.6.3** to **Figure.6.8.** Optimized chromatographic conditions for the determination of NIT and PHE were furnished in **Table.6.4.** Chromatograms of blank, NIT single, PHE single, standard and formulation were given from **Figure.6.9** to **Figure.6.13** respectively**.**

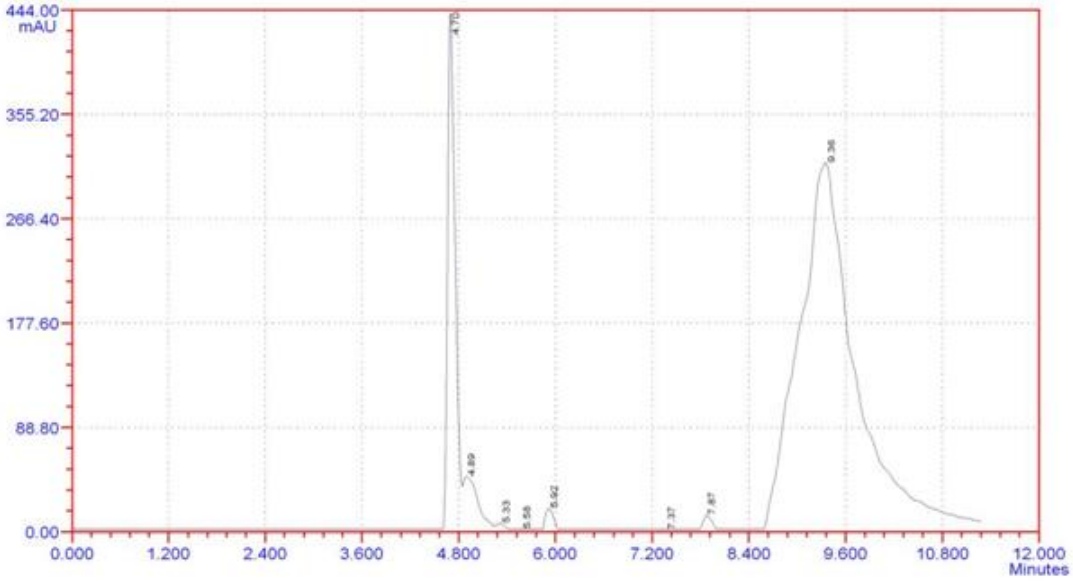
Table.6.3. Trial conditions of nirofurantoin and phenazopyridine

Trial	Mobile phase (v/v)	Wavelength	pH of mobile phase	Column	Flow rate
I	MeOH: ACN 50:50	248nm	5.4	Kromasil RP- C18	1.0ml/min
II	MeOH: ACN: Water 50:35:15	248nm	5.7	Hypersil RP- C18	1.0ml/min
III	MeOH: ACN 80:20	248nm	5.6	Hypersil BDS C18	1.0ml/min
IV	MeOH: Acetate Buffer 90:10	248nm	5.6	Hypersil BDS C18	1.0ml/min
V	MeOH: Acetate Buffer 75:25	248nm	5.0	Hypersil BDS C18	1.0ml/min
VI	MeOH: Acetate Buffer 75:25	248nm	4.8	Hypersil BDS C18	1.0ml/min



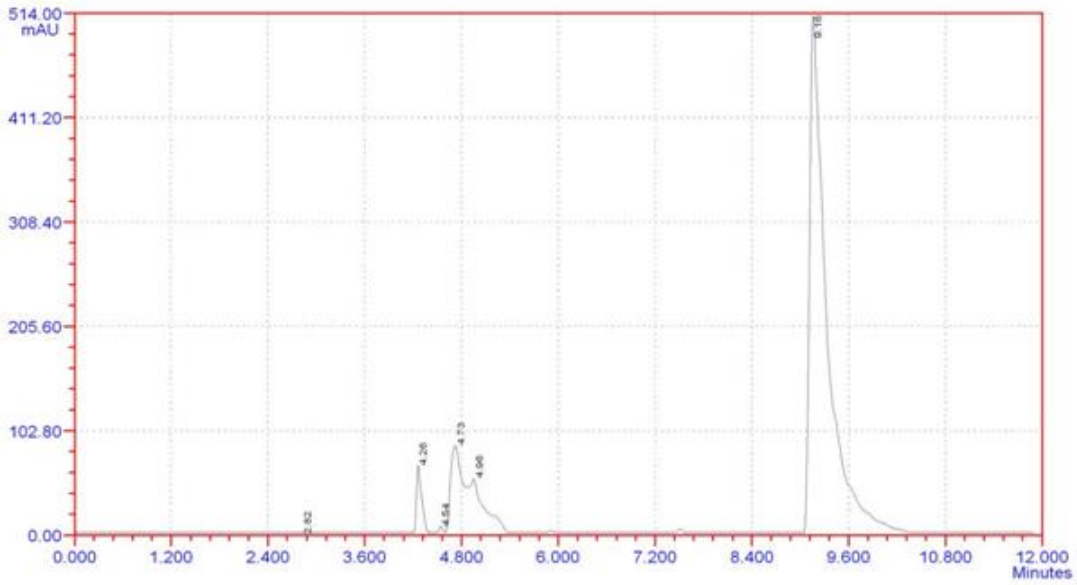
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.895	1135	28964.8	0.775	1.11	257
2		4.495	47209	471895.6	12.634	1.27	4030
3		4.757	39454	521918.0	13.973	1.81	2577
4		5.072	782	3662.5	0.098	2.66	23371
5		5.928	416	3747.5	0.100	1.35	8632
6		9.607	15021	1461987.9	39.142	0.53	194
7		9.793	14778	983538.7	26.333	10.78	432
8		11.323	11208	259346.9	6.944	1.05	4773
	Sum:		130003	3735061.8	100.0000		

Figure.6.3. Trial chromatogram I



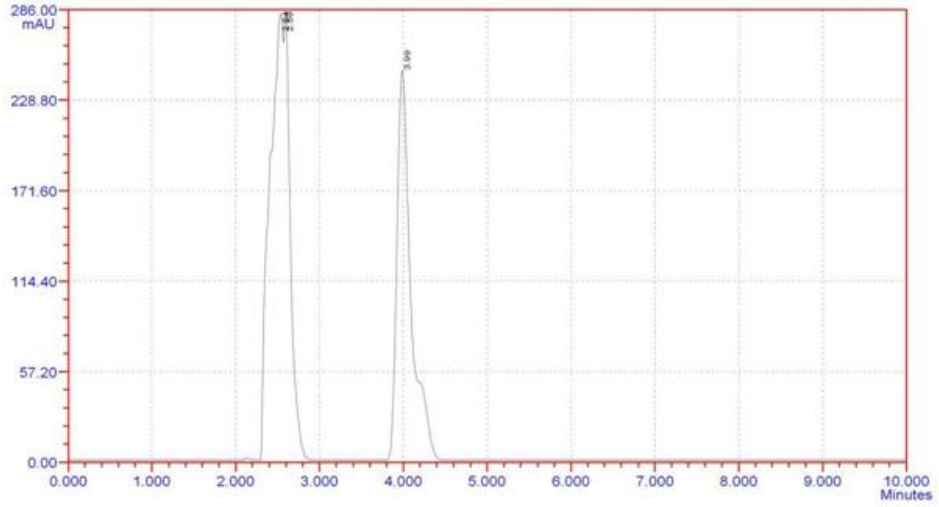
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.697	44445	293392.6	15.470	1.29	10091
2		4.892	4975	65344.7	3.446	4.74	2765
3		5.325	919	7667.9	0.404	1.63	8119
4		5.579	445	3978.6	0.210	1.23	7760
5		5.917	2194	16914.1	0.892	1.09	11741
6		7.372	443	4401.0	0.232	0.98	10975
7		7.869	1579	13717.3	0.723	1.15	16352
8		9.355	31219	1491098.7	78.623	1.76	765
	Sum:		86219	1896514.9	100.0000		

Figure.6.4. Trial chromatogram II



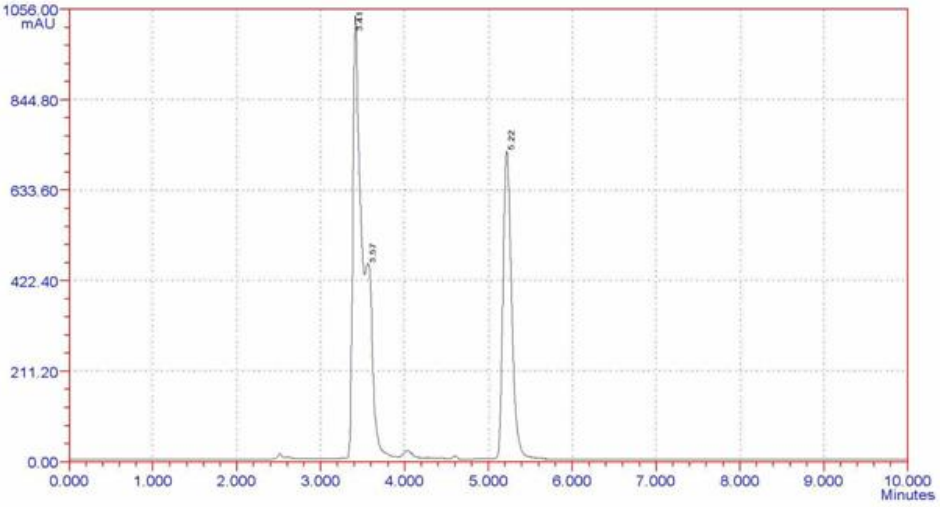
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.824	639	14419.3	1.380	1.09	312
2		4.255	7388	39017.1	3.733	1.42	12941
3		4.537	1334	9515.2	0.910	0.75	8064
4		4.730	9319	101822.8	9.741	1.09	3736
5		4.957	6016	91757.4	8.778	4.78	2105
6		9.157	51612	788719.9	75.457	5.60	7156
	Sum:		76308	1045251.6	100.0000		

Figure.6.5. Trial chromatogram III



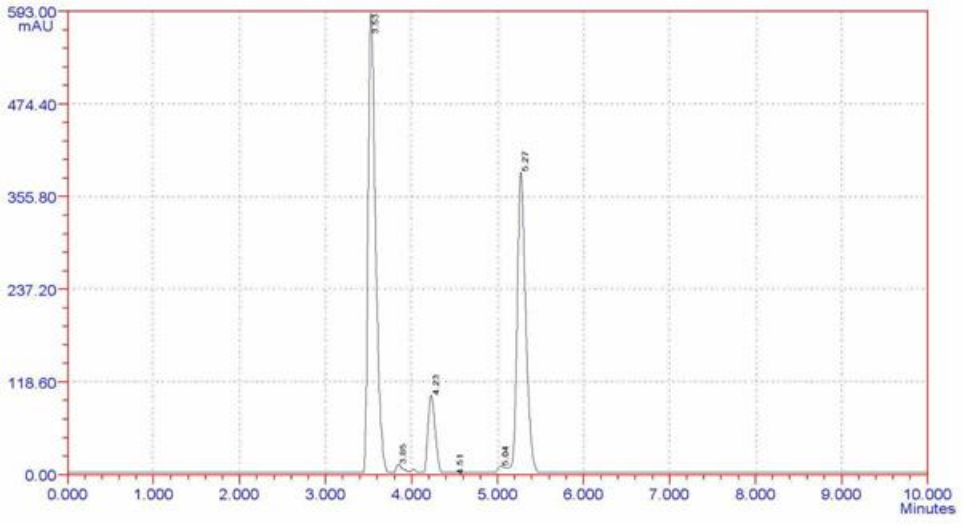
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.542	28442	304409.1	40.281	0.55	1124
2		2.592	28519	157265.2	20.810	7.06	4402
3		3.987	24835	294034.8	38.908	1.79	2260
	Sum:		81796	755709.2	100.0000		

Figure.6.6. Trial chromatogram IV



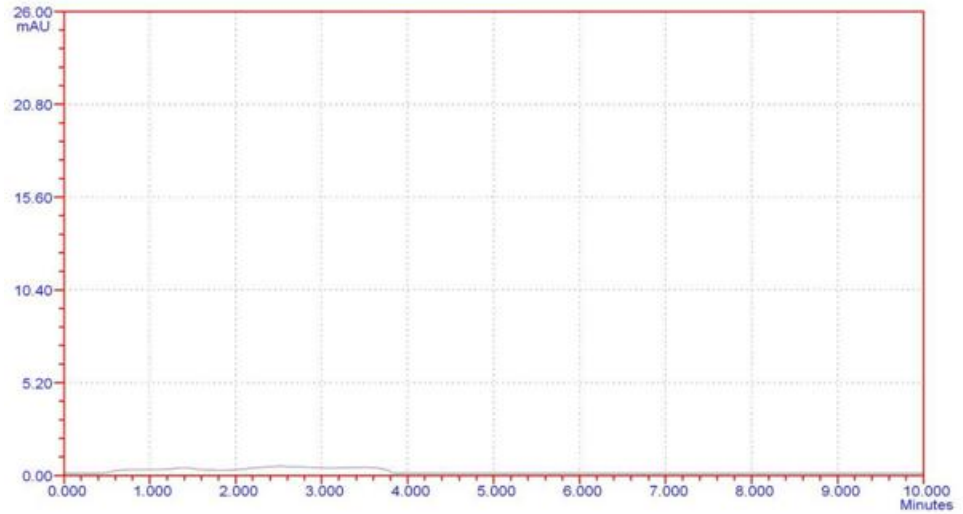
ID	Name	Retain.T	Height	Area	Conc	Half Width	Tail.Factor	Theo.Plate
1		3.413	103264	655373.5	45.068	6.35	1.16	5765
2		3.567	45295	283815.9	19.517	6.27	4.23	6458
3		5.220	71814	515004.4	35.415	7.17	1.71	10560
	Sum:		220373	1454193.8	100.0000			

Figure.6.7. Trial chromatogram V



ID	Name	Retain.T	Height	Area	Conc	Half Width	Tail.Factor	Theo.Plate
1		3.527	60065	376446.5	48.647	6.27	1.84	6311
2		3.847	1333	7256.7	0.938	5.44	1.68	9951
3		4.228	10663	68060.4	8.795	6.38	1.19	8747
4		4.508	315	4102.6	0.530	13.02	1.37	2388
1		5.037	2360	21071.5	2.723	8.93	0.73	6342
6		5.272	39762	296897.7	38.367	7.47	1.59	9934
	Sum:		114498	773835.3	100.0000			

Figure.6.8. Trial chromatogram VI

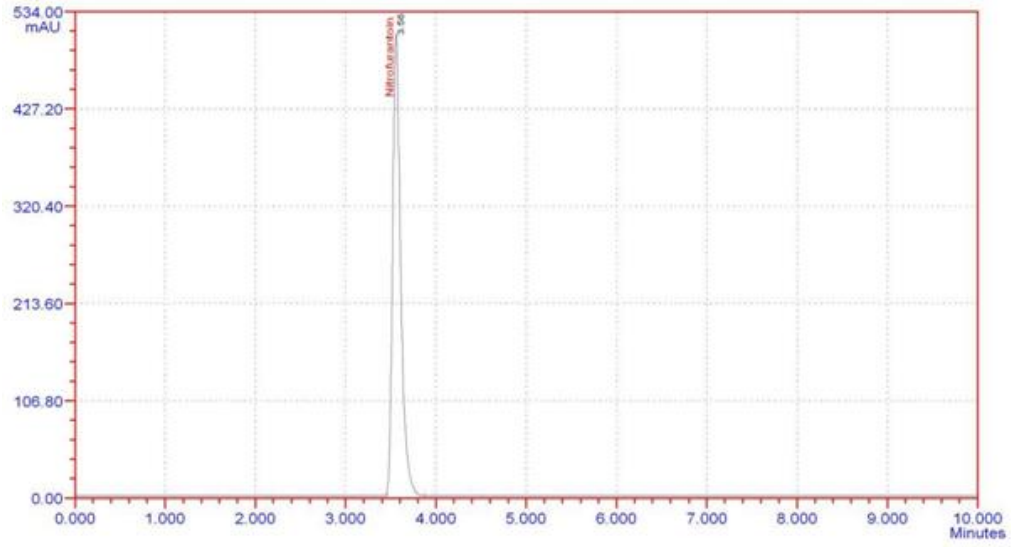


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
	Sum:		0	0.0	0.0000		

Figure.6.9. Chromatogram of blank of nitrofurantoin and phenazopyridine

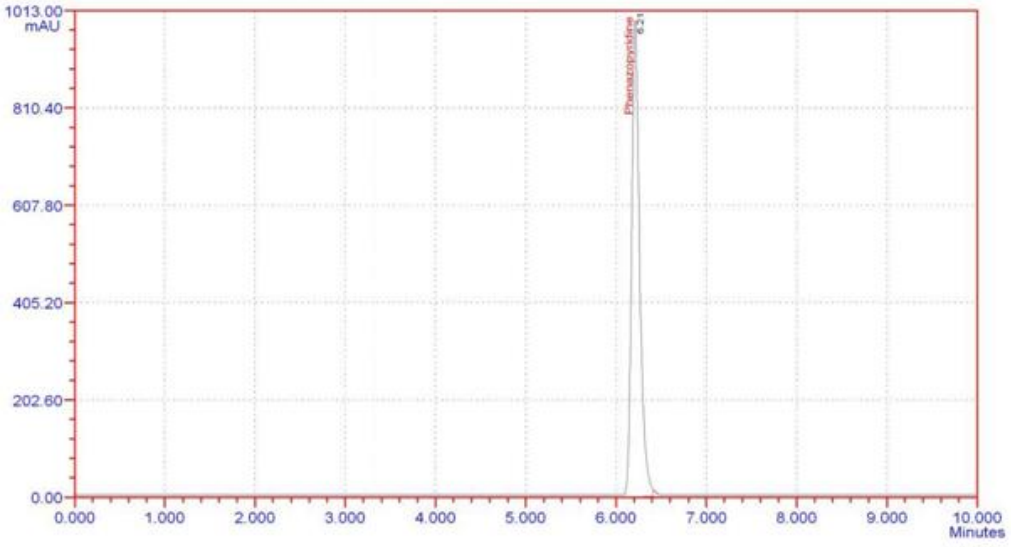
Table.6.4. Optimized chromatographic conditions of nitrofurantoin and phenazopyridine

1	Pump mode	Isocratic	
2	Column	C 18 (250X4.6 mm, 5µm)	
3	Injector	Rheodyne	
4	Injector Volume	20µl	
5	Diluent	Methanol	
6	Mobile phase	Methanol: Acetate Buffer in 75:25 (v/v)	
7	Pump pressure	10.7±5 MPa	
8	Mobile phase pH	4.5	
9	Wavelength	248nm	
10	Flow rate	1.0ml/min	
11	Run Time	10min	
12	Standard Concentration	Nitrofurantoin	20µg/ml
		Phenazopyridine	40µg/ml
13	Retention Time	Nitrofurantoin	3.56min
		Phenazopyridine	6.21min
14	Peak Area	Nitrofurantoin	323026
		Phenazopyridine	617610
15	Theoretical Plates	Nitrofurantoin	3291
		Phenazopyridine	19846
16	Tailing Factor	Nitrofurantoin	1.66
		Phenazopyridine	1.63



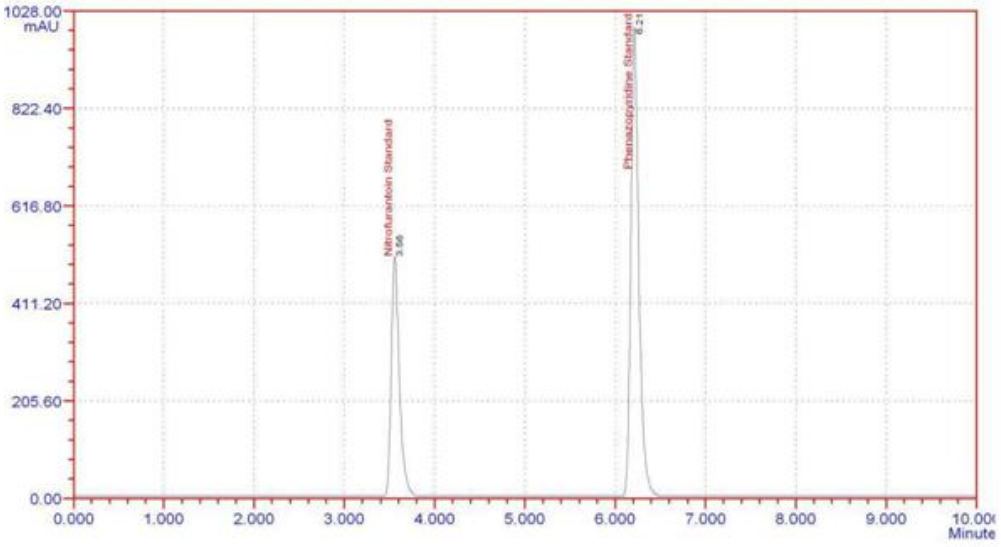
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Nitrofurantoin	3.560	50976	322472.0	100.000	1.82	6313
	Sum:		50976	322472.0	100.0000		

Figure.6.10. Chromatogram of nitrofurantoin single



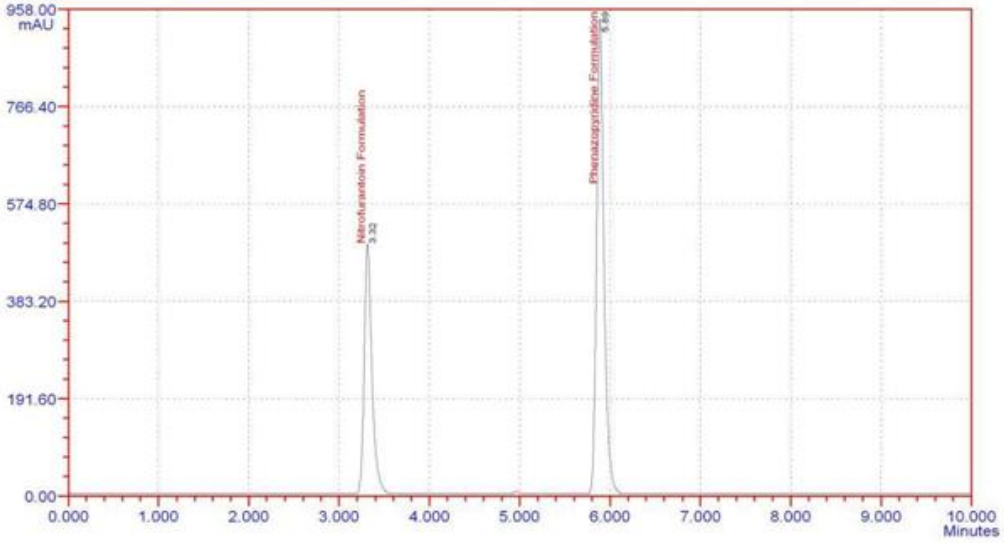
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Phenazopyridine	6.212	99214	613911.6	100.000	1.35	20085
	Sum:		99214	613911.6	100.0000		

Figure.6.11. Chromatogram of phenazopyridine single



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Nitrofurantoin Standard	3.560	50976	323026.8	33.941	1.66	6291	0.00
2	Phenazopyridine Standard	6.212	99214	617610.6	66.059	1.63	19846	14.90
	Sum:		150190	940637.5	100.0000			

Figure.6.12. Chromatogram of nitrofurantoin and phenazopyridine standard



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Nitrofurantoin Formulation	3.315	51385	320435.1	34.431	1.09	5634	0.00
2	Phenazopyridine Formulation	5.885	96896	610212.7	65.569	1.10	17407	14.47
	Sum:		148281	930647.8	100.0000			

Figure.6.13. Chromatogram of nitrofurantoin and phenazopyridine formulation

6.5. METHOD VALIDATION

6.5.1. Specificity

By the careful study of chromatograms of NIT and PHE, it was observed that, there was no interference of diluents and placebo in the analysis. This information strongly recommended the specificity of the method. Retention time of NIT and PHE were found to be 3.56min and 6.21min respectively.

6.5.2. System suitability

Results of system suitability were presented in **Table.6.5.**

Table.6.5. Results of system suitability of nitrofurantoin and phenazopyridine

S.No	Parameter	Nitrofurantoin	Phenazopyridine
1	API concentration	20µg/ml	40µg/ml
2	Retention Time	3.56min	6.21min
3	Resolution	-------	14.90
4	Peak Area	323026	617610
5	Theoretical Plates	3291	198436
6	Tailing Factor	1.66	1.63

6.5.3. Linearity

Results of linearity were tabulated in **Table.6.6.** Calibration curves were given in **Figure.6.14 & 6.15.** Correlation coefficient of NIT was 0.999 and that of PHE was 0.998.

Table.6.6. Results of linearity of nitrofurantoin and phenazopyridine

S.No	Nitrofurantoin		Phenazopyridine	
	Concentration in 20µg/ml	**Peak Area**	**Concentration in 40µg/ml**	**Peak Area**
1	5	151619	10	195206
2	10	208102	20	313400
3	15	269586	30	463792
4	20	323026	40	617610
5	25	391284	50	744801
6	30	448237	60	889912

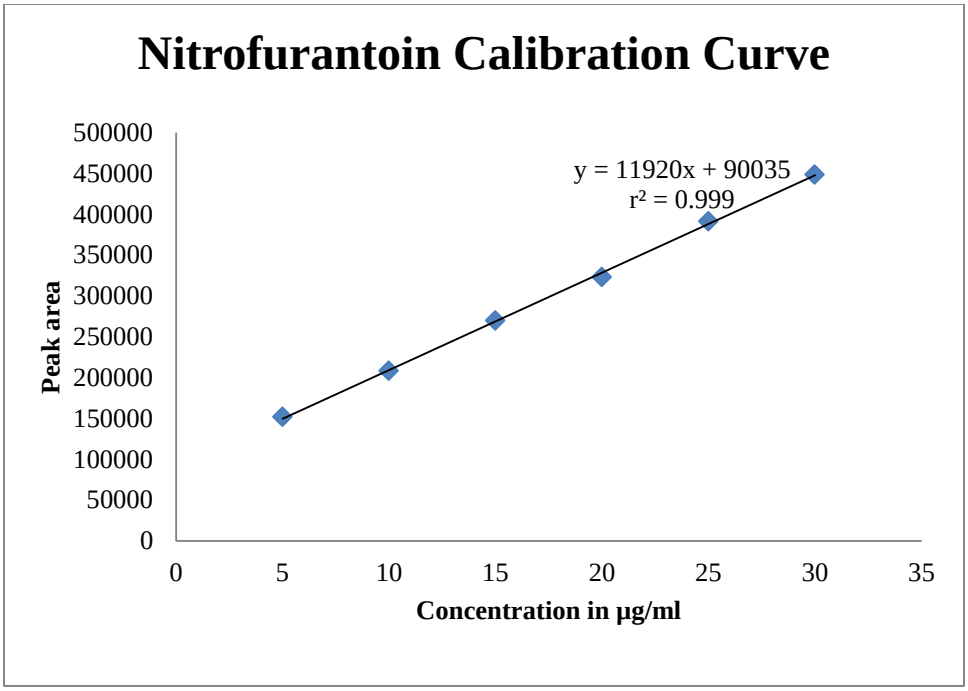


Figure.6.14. Calibration curve of nitrofurantoin

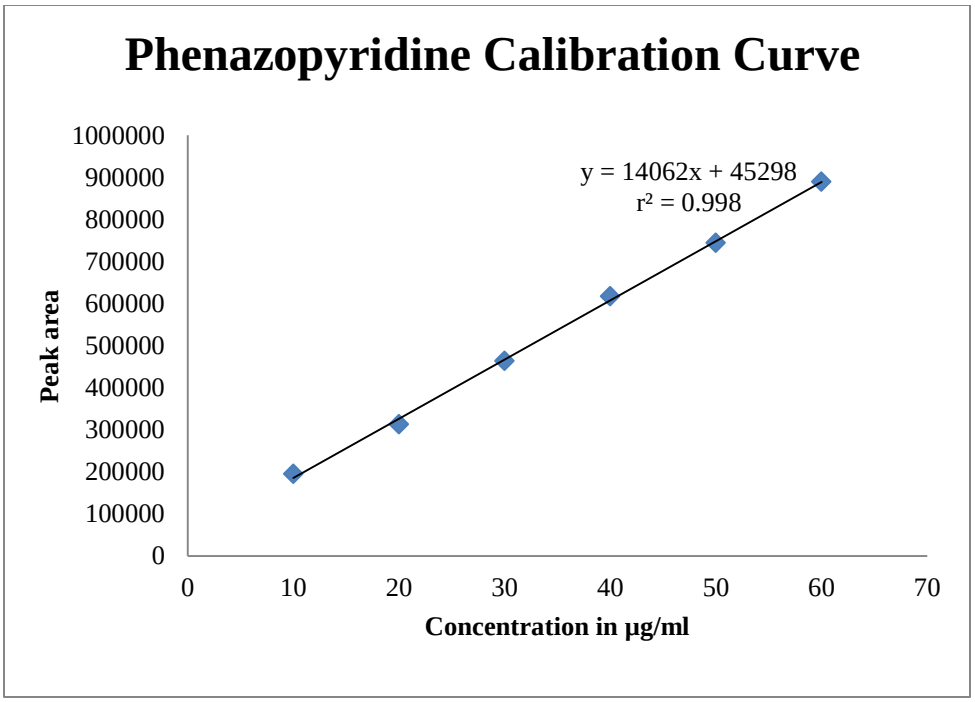


Figure.6.15. Calibration curve of phenazopyridine

6.5.4. Accuracy

Accuracy of the method was calculated through recovery studies. Recovery results of NIT and PHE were arranged in **Table.6.7 & 6.8** respectively.

6.5.5. Precision

Precision of the method was measured through intra-day precision and inter-day precision and the corresponding results were tabulated in **Table.6.9 & 6.10.**

6.5.6. Ruggedness

Ruggedness of the proposed method was measured through the study of reproducibility of NIT and PHE. Results of ruggedness were shown in **Table.6.11.**

6.5.7. Robustness

Robustness of the method was measured and the results were found to be within the proper limits. Results of robustness were shown in **Table.6.12**.

Table.6.7. Recovery results of nitrofurantoin

S.No	Spiked Level	Concentration in 20µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	10	5	15	14.899	99.329
2		10	5	15	14.814	98.763
3		10	5	15	14.959	99.724
4	100%	10	10	20	19.780	98.898
5		10	10	20	19.935	99.673
6		10	10	20	19.933	99.665
7	150%	10	15	25	25.412	101.650
8		10	15	25	25.163	100.650
9		10	15	25	25.223	100.891

Table.6.8. Recovery results of phenazopyridine

S.No	Spiked Level	Concentration in 40µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	20	10	30	29.726	99.088
2		20	10	30	29.684	98.945
s3		20	10	30	29.677	98.924
4	100%	20	20	40	39.433	98.584
5		20	20	40	39.897	99.743
6		20	20	40	39.678	99.195
7	150%	20	30	50	49.653	99.306
8		20	30	50	49.556	99.111
9		20	30	50	49.224	98.448

Table.6.9. Results of intra-day precision of nitrofurantoin and phenazopyridine

S. No	Nitrofurantoin at 20µg/ml	Phenazopyridine at 40µg/ml
1	328516	617802
2	329615	616819
3	323475	613872
4	325212	612730
5	327535	618274
6	325855	612232
%RSD	**0.696**	**0.433**

Table.6.10. Results of inter-day precision of nitrofurantoin and phenazopyridine

S. No	Nitrofurantoin at 20µg/ml	Phenazopyridine at 40µg/ml
1	325087	612813
2	324594	618528
3	323800	618675
4	324316	616333
5	322611	613626
6	326782	612882
%RSD	**0.428**	**0.445**

Table.6.11. Results of ruggedness of nitrofurantoin and phenazopyridine

S. No	Nitrofurantoin at 20µg/ml	Phenazopyridine at 40µg/ml
1	321539	614715
2	322136	609080
3	321494	609532
4	321380	611161
5	324451	609183
6	328066	614175
%RSD	**0.823**	**0.417**

Table.6.12. Results of robustness of nitrofurantoin and phenazopyridine

S.No	Condition	Change	Nitrofurantoin		Phenazopyridine	
			Area	% Change	Area	% Change
1	Standard	NO Change	323026	……..	617610	…….
2	MP 1	MeOH: Acetate Buffer 70:30 (v/v)	320967	0.637	613285	0.700
3	MP 2	MeOH: Acetate Buffer 80:20 (v/v)	326460	1.063	611038	1.064
4	WL 1	4.4	322182	0.261	616211	0.226
5	WL 2	4.6	325404	0.736	610495	1.152
6	pH 1	244nm	324849	0.564	614716	0.468
7	pH 2	252nm	321272	0.543	610545	1.144

6.5.8. Limit of detection and limit of quantitation

LOD and LOQ values of NIT and PHE were specified in **Table.6.13**.

6.5.9. Solution stability

Solution stability of the standard solution of NIT and PHE was determined to be 24hr. Results of solution stability were arranged in **Table.6.14.**

Table.6.13. Results of LOD and LOQ of nitrofurantoin and phenazopyridine

Drug	LOD	LOQ
Nitrofurantoin	0.02µg/ml	0.07µg/ml
Phenazopyridine	0.04µg/ml	0.15µg/ml

Table.6.14. Results of solution stability of nitrofurantoin and phenazopyridine

S.No	Time in Hours	Nitrofurantoin		Phenazopyridine	
		Area	% Assay	Area	% Assay
1	1.0	324390	100.4223	608741	98.56398
2	2.0	322690	99.89598	614839	99.55133
3	4.0	321502	99.52821	608881	98.58665
4	8.0	321814	99.6248	612468	99.16744
5	12.0	321380	99.49044	608728	98.56188
6	24.0	317175	98.18869	605559	98.04877

6.5.10. Formulation

Results of NIT and PHE formulation were presented in **Table.6.15.** The percentage assay of was found to be 99.365 for NIT and 98.529 for PHE.

Table.6.15. Results of nitrofurantoin and phenazopyridine formulation

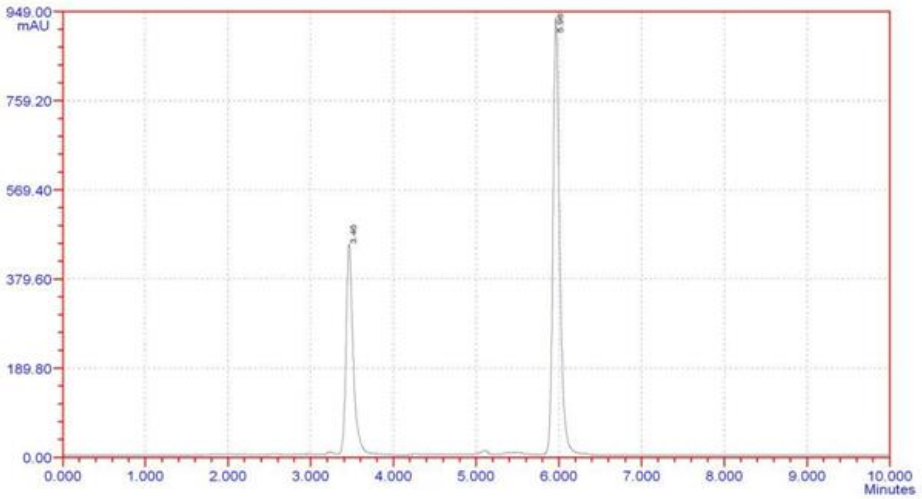
S.No	Drug	Brand	Dosage	Amount	Amount	%Assay
1	Nitrofurantoin	Nephrogesic	10mg	20µg/ml	19.839µg/ml	99.198
2	Phenazopyridine		20mg	40µg/ml	39.52µg/ml	98.802

6.6. FORCED DEGRADATION STUDIES

Sample solutions were exposed to various stress conditions, such as acidic, basic, peroxide, thermal, light and UV light, to measure the stability of NIT and PHE formulation. The number of degradation peaks identified in each and every degradation condition were presented in **Table.6.16**. Degradation chromatograms were shown from **Figure.6.16 to 6.22**.

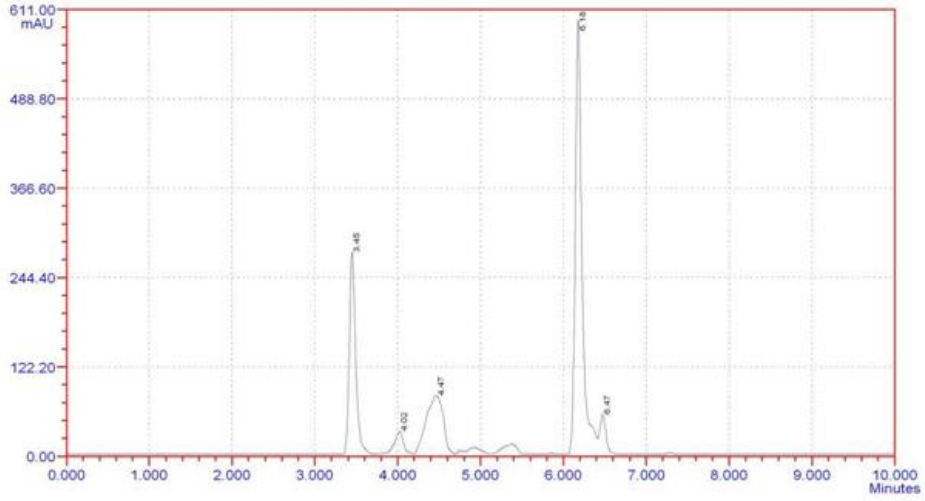
Table.6.16. Forced degradation studies of nitrofurantoin and phenazopyridine

S. No	Condition	No of degradation Peaks Observed
1	Aqueous	0
2	Acidic	3
3	Basic	1
4	Peroxide	1
5	Thermal	3
6	Sun light	1
7	UV light	2



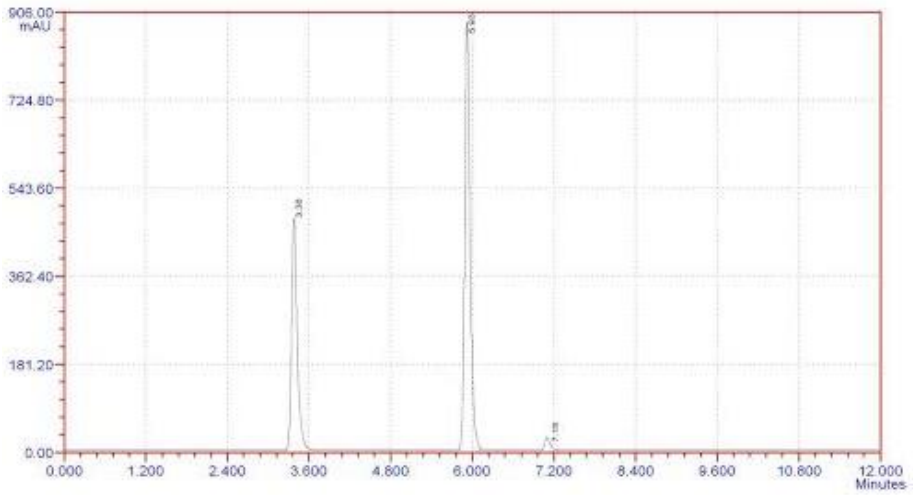
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.462	44572	263134.5	32.489	0.10	6853	0.00
2		5.963	92617	525585.6	67.511	1.51	22008	15.25
	Sum:		137189	788720.0	100.0000			

Figure.6.16. Chromatogram of aqueous degradation



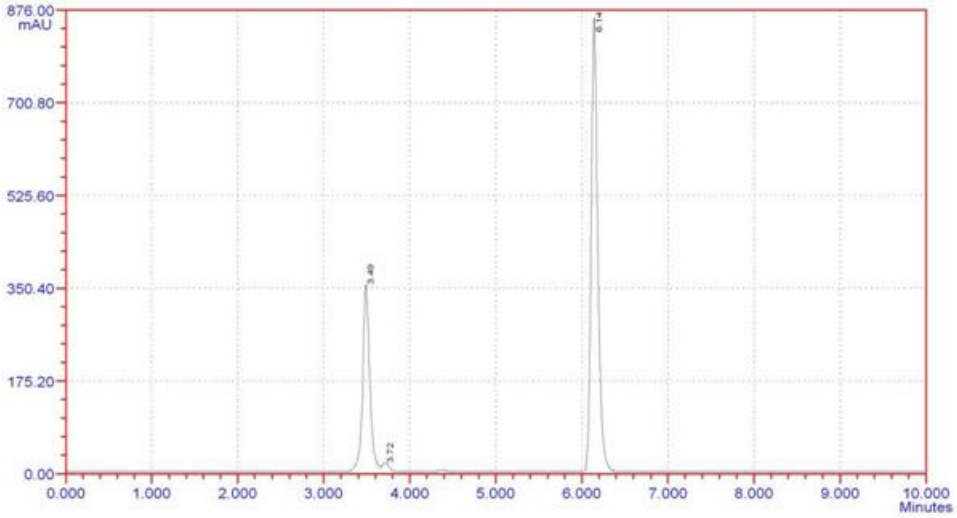
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.448	27708	149388.4	22.512	1.72	8151	0.00
2		4.023	2929	21189.5	3.193	1.01	6163	3.21
3		4.468	8157	118217.7	17.815	0.91	1894	1.45
4		6.178	59509	343987.2	51.837	1.58	22768	5.95
5		6.475	5581	30813.8	4.643	2.10	27410	1.85
	Sum:		103884	663596.6	100.0000			

Figure.6.17. Chromatogram of acid degradation



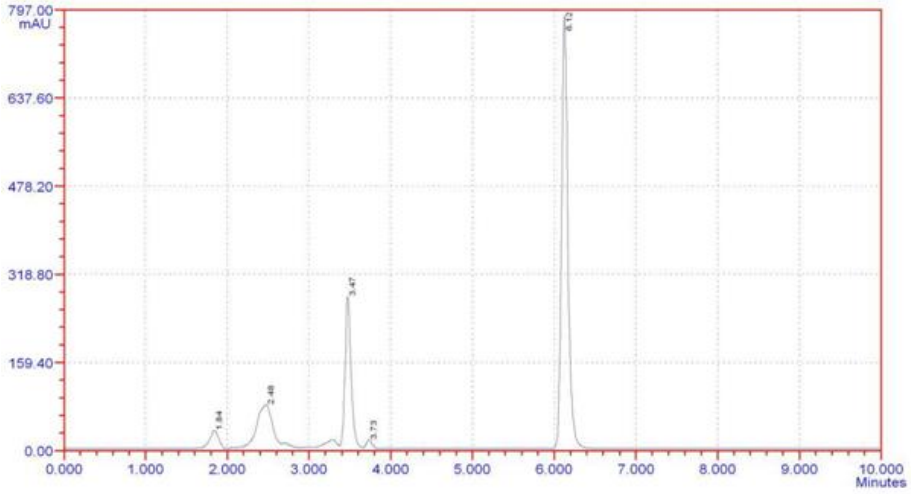
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.382	50968	323360.2	32.920	1.20	5663
2		5.925	94512	605599.5	61.654	1.14	17041
3		7.155	2653	53289.3	5.425	0.50	2529
	Sum:		148133	982249.0	100.0000		

Figure.6.18. Chromatogram of base degradation



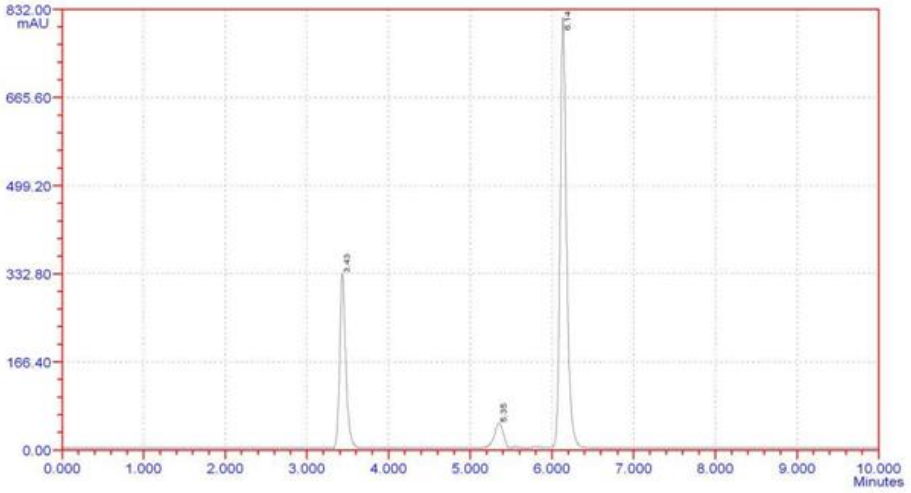
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.490	35369	203326.7	28.767	0.95	7347	0.00
2		3.717	1696	10499.1	1.379	1.56	7185	1.34
3		6.143	85884	462547.3	69.853	1.49	25934	14.80
	Sum:		122949	676373.1	100.0000			

Figure.6.19. Chromatogram of peroxide degradation



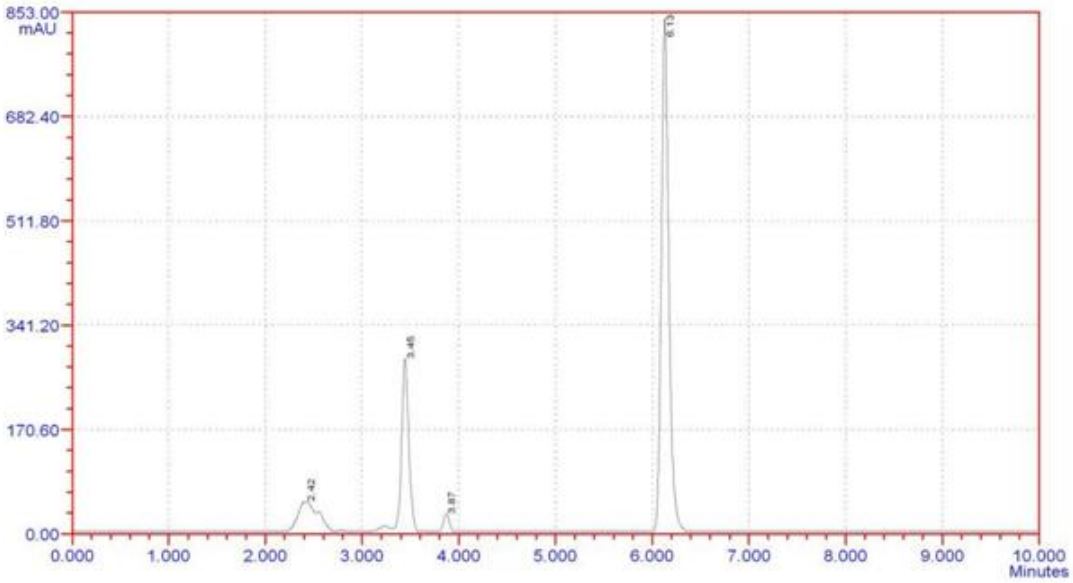
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.843	3352	25153.2	2.849	0.93	1203	0.00
2		2.475	7376	96403.5	6.268	0.88	715	2.17
3		3.472	27145	135630.7	23.068	1.22	9622	3.89
4		3.733	1572	7525.2	1.336	1.09	12122	1.89
5		6.123	78229	423009.4	66.479	1.49	25560	16.55
	Sum:		117674	687722.1	100.0000			

Figure.6.20. Chromatogram of thermal degradation



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.433	33157	173381.0	26.747	1.48	8590	0.00
2		5.351	4690	34363.9	5.301	0.88	10631	10.79
3		6.136	81212	440473.8	67.951	1.41	25511	4.35
	Sum:		119059	648218.8	100.0000			

Figure.6.21. Chromatogram of light degradation



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		2.420	5236	82337.7	4.336	1.44	472	0.00
2		3.445	28308	145934.4	23.440	0.97	8901	3.47
3		3.872	3108	15208.0	2.574	0.99	12477	3.00
4		6.130	84114	453953.2	69.650	1.49	25713	15.49
	Sum:		120766	697433.3	100.0000			

Figure.6.22. Chromatogram of UV light degradation

6.7. RESULTS AND DISCUSSION

A simple, sensitive, accurate and precise stability indicating RP-HPLC method was developed for the simultaneous estimation of NIT and PHE. On spectrophotometer, both the drugs NIT and PHE exhibited maximum absorption at a wavelength of 248nm. After several trials, hypersil BDS RP-C18 column was chosen for the separation and simultaneous estimation of NIT and PHE. Appropriate mobile phase was selected after testing mixtures of commonly used solvents like water, methanol and acetonitrile with or without the use of buffer in different combinations. Mobile phase consisting of methanol and acetate buffer (75:25, v/v) at a pH of 4.5 showed optimum separation and thus entire analysis was performed at these conditions only. Optimum chromatographic conditions were furnished in **Table.6.4**.

Measurement of specificity of the developed method initiated the method of validation of NIT and PHE. Retention times reported for NIT and PHE were 3.56min and 6.21min respectively. Various properties of chromatogram were studied especially tailing factor (<2.0), resolution (>2.0) and theoretical plates (> 2000) and were given in **Table.6.5.** Absence of additional peaks in the chromatograms of NIT single **(Figure.6.10),** PHE single **(Figure.6.11),** NIT and PHE standard **(Figure.6.12)** and NIT and PHE formulation **(Figure.6.13)** revealed the non-interference of excipients in the analysis. Linearity **(Table.6.6)** was found to good in the concentration range of 5-30µg/ml for NIT and was 10-60µg/ml for PHE. Regression equation of NIT and PHE was $y = 11920x + 900.35$ ($r^2 = 0.999$) and $y = 14062x + 45298$ ($r^2 = 0.998$) respectively. Calibration curves of NIT and PHE were given in **Figure.6.14 & Figure.6.15.**

Recovery studies at three different concentration levels especially 50%, 100% and 150% were tested and percentage RSD values emphasized the accuracy of the method. Recovery results of NIT and PHE were specified in **Table.6.7& 6.8** respectively. Precision of the method was studied by the repeated injection of standard solution of NIT

and PHE. Results of intra-day precision **(Table.6.9)** and inter-day precision **(Table.6.10)** proved the proposed method to be precise. Ruggedness of the method was measured on three different days by different analyst and the results were given in **Table.6.11**. Robustness of the method was determined with respect to small changes introduced in chromatographic parameters (wavelength, pH and mobile phase) and the results were shown in **Table.6.12.**

The method was recognized to be sensitive from the lowest values **(Table.6.13)** of LOD and LOQ. Stability study (**Table.6.14**) was conducted over a period of 248hr and it was reported that the solution was stable up to 24hr. The validated method was applied for the assay of commercial formulation of NIT and PHE. The results were tabulated in **Table.6.15**. Hence, the proposed method is applicable to commercial formulation for the estimation of NIT and PHE.

Various stress conditions such as acidic, basic, aqueous, peroxide, thermal, light and UV light were applied for inducing stress in the molecule. The results were tabulated in **Table.6.16**. Degradation studies are highly useful in investigating degradation path ways and degradation products which may arise during storage**.** Hence the forced degradation studies provide valuable information that is highly advantageous during the manufacturing, formulation development and packing of NIT and PHE in pharmaceutical formulation.

6.8. CONCLUSIONS

The aim of the present investigation was to develop an isocratic HPLC method for the simultaneous analysis of NIT and PHE in the pharmaceutical dosage forms. The most significant feature of the proposed method was time and the two drugs were analysed in a time period of less than 7min. Addition of forced degradation studies extended the range of the proposed method. Different stress conditions were applied to the standard solution of NIT and PHE to measure the stability of the molecule. Thus the proposed method can be used conveniently for the regular estimation of NIT and PHE in the tablet dosage forms.

Future research must concentrate on investigating degradation path ways, degradation product and secondary degradation products.

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