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Separation and determination of synthetic impurities of norfloxacin by reversed-phase high performance liquid chromatography☆

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Abstract

A simple and rapid high performance liquid chromatographic method for the separation and determination of synthetic impurities of norfloxacin was developed. The separation was achieved on a reversed-phase C_{18} column using 0.01 M potassium dihydrogen orthophosphate and acetonitrile (60:40, v/v, pH 3.0) as mobile solvent at a flow rate of 1.0 ml/min at 40 °C and a UV detection at 260 nm. The method was used not only for quality assurance but also for monitoring the chemical reactions during the process development work in the laboratory. It was found to be specific, precise and reliable for determination of unreacted levels of raw materials, intermediates and the finished products of norfloxacin.

Keywords: Norfloxacin; Process impurities; Reversed-phase HPLC

1. Introduction

Norfloxacin (1-ethyl-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinly)-3-quinoline carboxylic acid) is a broad spectrum antibiotic, effective against both gram positive and gram negative organisms including: *Pseudomonas gonoccoci*, *H. influenzae*, *staphylococci*, *streptococci* and used in the treatment of urinary, respiratory tract infections, gastro-intestinal and sexually transmitted diseases [1,2]. It is synthesized by heating 1-ethyl-6-fluoro-7-chloro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (ECA) with piperazine in a laboratory [3]. During this reaction, not only the unreacted ECA but also its related analogues viz., 7-chloro-6-fluoro-1-methyl-4-oxo-1,4-dihydro-3-qui-nolinecarboxylic acid (MCA) and ethyl-7-chloro-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylate (CAT) may be carried over in small quantities in to the bulk products of NOR, thus reducing its quality and quantity significantly. Therefore, the separation and determination of synthetic impurities of NOR is of great importance not only for quality control but also monitoring of reactions during process development of fluoroquinoline anti-microbial agents.

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Several HPLC methods for determination of NOR in pharmaceuticals as well as biological fluids using ultra-violet and fluorescence detection have been reported in the literature [4–8]. Most of these methods either involve the use of a gradient elution or previous acylation of NOR for determination of the active ingredient in a variety of sample matrices. A number of spectrophotometric and polarographic procedures including adsorptive differential pulse stripping voltametry of NOR were studied [9–12]. These procedures are not only tedious for removal of excipients from tablets but also lack simplicity and specificity. A thorough literature search has revealed that, no method was reported for separation and determination of the process related impurities of NOR. Borrego et al. have studied the photo-stability of NOR contained in directly compressible tablets and estimated the closely related ethylenediamine degradate by HPLC [13]. HPTLC method for monitoring the NOR residues on pharmaceutical equipment using a fluorescence detector was also reported [14]. In this paper, we describe a simple and rapid HPLC method for separation and determination of process components of NOR in bulk drugs and formulations using a reversed-phase C_{18} column and 0.01 M potassium dihydrogen orthophosphate-acetonitrile (60:40, v/v; pH 3.0 ± 0.3) as eluent at 40 °C temperature.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical reagent grade unless stated other-wise. Potassium dihydrogen orthophosphate (E. Merck, Mumbai, India) and HPLC grade acetonitrile obtained from Qualigens, Mumbai, India, was used. Glass distilled water and deionized water (Nanopure, Barnsted, USA) was used throughout the study. NOR and its synthetic impurities were gifted by Metropolitan Overseas Limited, Hirehalli, Tumkur, India.

2.2. Apparatus

A HPLC system composed of two LC-10 AT VP pumps, an SPD-M 10A VP diode array detector an SIL-10AD VP auto injector, a DGU 12 A degasser and SCL-10 VP system controller (all from Shimadzu, Kyoto, Japan). A reversed-phase symmetry C_{18} (Waters, Milford, USA) column (25 cm × 4.6 mm i.d., particle size 5 μm) was used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

The mobile phase was 0.01 M potassium dihydrogen orthophosphate (adjusted to pH 3.0 ± 0.3 with phosphoric acid) and acetonitrile (60:40, v/v). Before delivering in to the system it was filtered through 0.45 μm PTFE filter and degassed using vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at 40 °C temperature. Chromatograms were recorded at 260 nm using diode array detector.

2.4. Analytical procedure

Samples (10 mg) were accurately weighed and taken in 100 ml volumetric flask. One milliliter of 0.1N NaOH was added to the flask. After dissolving the samples the volume was made up to the mark with the mobile phase. Similarly NOR standard (10 mg) in 100 ml was prepared. A 20 μl volume of each sample was injected and chromatographed under the above conditions. Synthetic mixtures containing NOR, MCA, ECA, CAT bulk drugs and formulations were analyzed under identical conditions. The amount of impurities were calculated from their respective peak areas.

3. Results and discussion

Fig. 1 shows the molecular structures of NOR and its structurally related-synthetic impurities viz., CAT, ECA and MCA studied in the present investigation. All these materials were subjected to separation by reversed-phase HPLC. The separation and resolution were found to be pH dependent. Due care was given to the pH of the mobile phase while standardizing the HPLC conditions. pH values between 3.0 and 5.0 were found to be the optimum values for good separation. Acetonitrile was used an organic solvent modifier

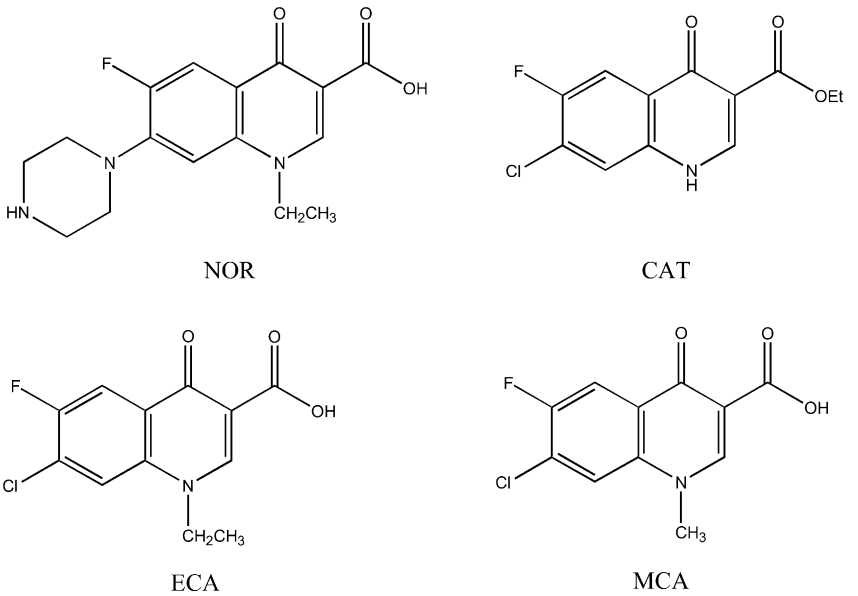

Fig. 1. Norfloxacin and its related impurities.

to improve the separation. The effect of concentration of acetonitrile and temperature of the column on resolution was also studied. The chromatographic separation was also found to be dependent on the concentration of acetonitrile and the separation was found to be optimum at 40% (v/v). The retention of NOR, CAT, ECA and MCA as function of pH, temperature and concentration of acetonitrile is shown in Fig. 2. The optimum resolution between the compounds of interest was obtained at 40 °C using Symmetry C_{18} column with aqueous 0.01 M potassium dihydrogen orthophosphate-acetonitrile (60:40, v/v; pH 3.0) as eluent. A typical chromatogram of a synthetic mixture containing NOR, CAT, ECA and MCA is shown

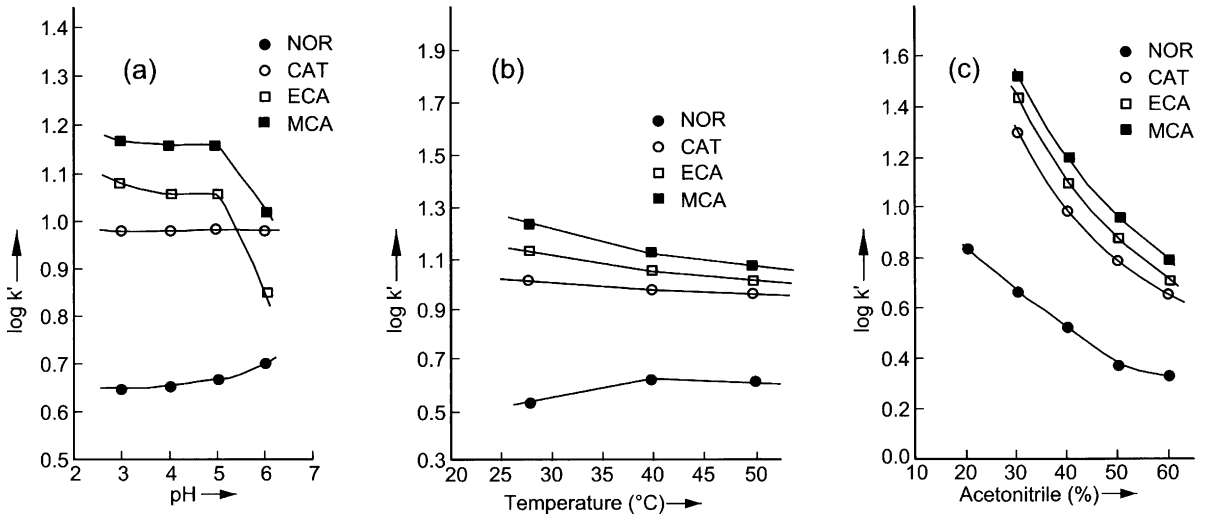

Fig. 2. Effect of (a) pH, (b) temperature and (c) concentration of acetonitrile on retention of NOR and its impurities.

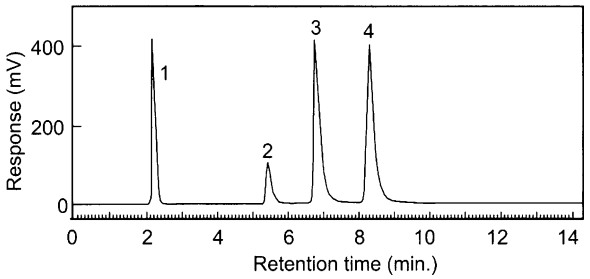

Fig. 3. Typical chromatogram of a mixture containing 50 μg each of (1) NOR, (2) CAT, (3) ECA and (4) MCA.

in Fig. 3. The peaks were identified by injecting and comparing the retention times with those of authentic standards. Reproducible peak shapes were obtained under the optimum conditions. The peak tailing factors were calculated for NOR and its impurities and are given in Table 1. From these values, it could be clearly seen that the shapes of these peaks are undistorted. Therefore, the symmetry C_{18} column was preferred over other columns viz., spehrisorb C_{18} and CN, because it has provided better resolution of the peaks with symmetry and reproducibility. The retention time (t_R), relative retention time (RRT), number of theoretical plates (N), relative response factors (RRF) and wavelengths of maximum absorption (λ_{max}) were determined and recorded in Table 1. The UV detector was set at 260 nm for both detection and quantification. This was selected based on the observations that the response of the chromatographic peaks of NOR and its impurities were better when compared to the determinations made at other wavelengths.

3.1. Accuracy and precision

Standard mixtures containing known amounts of NOR, MCA, ECA and CAT were prepared and analyzed by HPLC. The accuracy of the method was checked for three different concentration levels by standard addition technique. Small quantities of impurities were added to the sample and chromatographed. It was found that these additions were accurately reflected in their peak areas. All estimations were repeated thrice and standard deviations (S.D.) were calculated (Table 2). The precision of the method was determined (R.S.D. 0.89%) on replicate injections ($n = 5$) of a standard solution of NOR and reported.

Table 2
Accuracy data for standard mixtures containing NOR, CAT, ECA and MCA

Compound	Taken ($\times 10^{-6}$ g)	Found ($\times 10^{-6}$ g) mean ± S.D.	Recovery (%)
NOR	1.02	1.04 ± 0.03	101.96
	25.08	25.21 ± 0.45	100.51
	50.05	50.48 ± 0.72	100.86
CAT	3.04	2.94 ± 0.04	96.71
	6.02	5.90 ± 0.12	98.00
	10.05	9.86 ± 0.35	98.11
ECA	2.01	2.06 ± 0.03	102.49
	12.05	12.29 ± 0.32	101.99
	25.03	25.31 ± 0.44	101.12
MCA	2.02	2.09 ± 0.03	103.47
	12.04	12.32 ± 0.35	102.32
	25.05	25.43 ± 0.46	101.52

3.2. Specificity

To demonstrate the specificity of the method two types of experiments were carried out. In the first experiment NOR bulk drug was spiked with known quantities of potential impurities. All the impurities were clearly separated and are not interfering with the retention time of NOR (Fig. 4). In another experiment, commercial formulations of NOR obtained

Table 1
Retention and response data for NOR and its impurities

Compound	Abbreviation	t_R (nm)	RRT	N	T_f	RRF	λ_{max} (min)
Norfloxacin	NOR	2.38	1.00	2828	1.02	1.00	279
Ethyl-7-chloro-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylate	CAT	5.48	2.30	6416	1.32	0.43	251
7-Chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid	ECA	6.83	2.87	8349	1.48	1.63	259
7-Chloro-6-fluoro-1-methyl-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid	MCA	8.32	3.50	8550	1.54	1.86	262

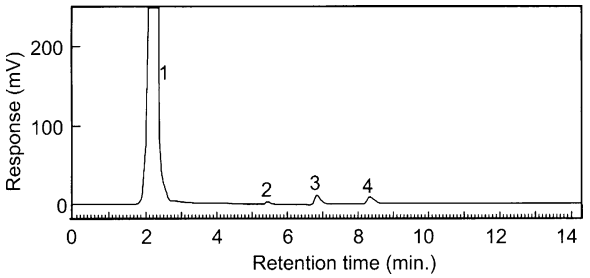

Fig. 4. Typical chromatogram of (1) NOR (200 μg) spiked with 2.0 μg each of (2) CAT, (3) ECA and (4) MCA.

from three different manufacturers were analyzed. The placebo analysis was also carried out and found that the excipients do not interfere either with NOR or any of the impurities (Fig. 5). This indicates the method is specific for the separation and determination of NOR and its process impurities in both bulk drugs and formulations.

3.3. Linearity

Calibration graphs (concentration vs. peak area) were constructed at six concentrations levels for NOR (1.0×10^{-6} to 50×10^{-6} g), MCA (2.0×10^{-6} to 25×10^{-6} g), ECA (2.0×10^{-6} to 25×10^{-6} g) and CAT (3.0×10^{-6} to 10×10^{-6} g) were studied. Three independent determinations were carried out at each concentration level. Good linearity was found between the mass integral response for each of the compound examined. Table 3 gives linearity equation, mass range and correlation coefficients for all compounds.

3.4. Stability

To determine the stability of NOR in the mobile phase, the drug was stored in the mobile phase for 24 h and chromatographed on the next day. The solutions were stable during the investigated 24 h and observed that there is no degradation/increase in the percentage of impurities, i.e. no significant change was observed. Replicate injections ($n = 5$) of NOR solutions were performed and the relative standard deviation of peak area was determined with 1.53–1.89%. In another experiment, the stability of NOR in the 0.1N NaOH was also tested. The bulk drug was dissolved in the 0.1N NaOH and stored for 24 h with out adding the mobile phase and chromatographed on the next day. The chromatograms were shown in Fig. 6. It could be seen from the Fig. 6 that there is no degradation/increase in the percentage of impurities of

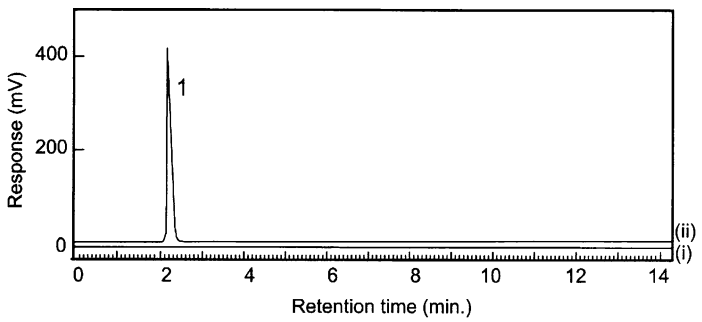

Fig. 5. Typical chromatogram of a (i) placebo and (ii) commercial formulation of NOR.

Table 3
Linear regression data for NOR and its impurities

S. no.	Compound	Mass range ($\times 10^{-6}$ g)	Linear regression	Correlation coefficient (r)	LOD ($\times 10^{-6}$ g)	LOQ ($\times 10^{-6}$ g)
1	NOR	1–50	$51556x - 4225.6$	0.9999	0.23	0.72
2	CAT	3–10	$22063x - 7630$	0.9995	0.80	2.42
3	ECA	2–25	$95448x - 62338$	0.9982	0.20	0.62
4	MCA	2–25	$107901x - 58626$	0.9990	0.20	0.62

Table 4
Robustness data for NOR and its impurities

Parameter	NOR		CAT		ECA		MCA	
	RRT	k'	RRT	k'	RRT	k'	RRT	k'
(a)								
Mobile phase composition (ACN, %)								
35	1.07	4.10	3.27	14.56	4.46	20.22	5.40	24.70
40	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
45	0.83	2.96	1.83	7.70	2.37	10.26	2.86	12.62
Mean ± S.D.	0.97 ± 0.03	3.61 ± 0.03	2.47 ± 0.04	10.74 ± 0.03	3.23 ± 0.02	14.38 ± 0.04	3.92 ± 0.03	17.65 ± 0.03
Ionic strength of KH_2PO_4 (M)								
0.008	0.98	3.66	2.28	9.86	2.84	12.52	3.49	15.60
0.010	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
0.012	1.03	3.90	2.32	10.04	2.89	12.77	3.52	15.74
Mean ± S.D.	1.00 ± 0.02	3.77 ± 0.04	2.30 ± 0.01	9.95 ± 0.02	2.87 ± 0.03	12.65 ± 0.03	3.50 ± 0.02	15.66 ± 0.02
pH								
2.7	0.98	3.66	2.30	9.96	2.90	12.82	3.52	15.74
3.0	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
3.3	1.01	3.80	2.30	9.96	2.84	12.52	3.48	15.56
Mean ± S.D.	1.00 ± 0.02	3.74 ± 0.03	2.30 ± 0.01	9.96 ± 0.01	2.87 ± 0.02	12.67 ± 0.04	3.50 ± 0.02	15.65 ± 0.03
Sample diluent volume of NaOH (ml)								
0.9	0.99	3.72	2.29	9.90	2.84	12.52	3.47	15.52
1.0	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
1.1	1.02	3.86	2.31	10.00	2.90	12.80	3.54	15.86
Mean ± S.D.	1.00 ± 0.01	3.78 ± 0.02	2.30 ± 0.01	9.95 ± 0.01	2.87 ± 0.02	12.66 ± 0.03	3.50 ± 0.02	15.67 ± 0.03
Temperature (°C)								
37	0.96	3.56	2.34	10.16	3.00	13.30	3.71	16.68
40	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
43	0.99	3.70	2.29	9.88	2.81	12.38	3.42	15.26
Mean ± S.D.	0.98 ± 0.02	3.67 ± 0.03	2.31 ± 0.02	10.00 ± 0.04	2.89 ± 0.03	12.78 ± 0.04	3.54 ± 0.02	15.86 ± 0.03
Flow rate (ml/min)								
0.9	1.01	3.80	2.32	10.02	2.89	12.78	3.52	15.74
1.0	1.00	3.76	2.30	9.96	2.87	12.66	3.50	15.64
1.1	0.97	3.64	2.28	9.84	2.85	12.76	3.49	15.60
Mean ± S.D.	0.99 ± 0.02	3.73 ± 0.03	2.30 ± 0.02	9.94 ± 0.02	2.87 ± 0.02	12.73 ± 0.03	3.50 ± 0.01	15.66 ± 0.02
(b)								
Mobile phase composition (ACN, %)								
35	1.08	4.20	3.31	14.96	4.51	20.74	5.41	25.08
40	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
45	0.84	3.04	1.86	7.96	2.42	10.66	2.87	12.84
Mean ± S.D.	0.97 ± 0.02	3.69 ± 0.03	2.50 ± 0.02	11.05 ± 0.03	3.28 ± 0.02	14.79 ± 0.04	3.94 ± 0.03	17.98 ± 0.05
Ionic strength of KH_2PO_4 (M)								
0.008	0.98	3.72	2.31	10.14	2.86	12.78	3.52	15.96
0.010	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
0.012	1.02	3.94	2.36	10.38	2.93	13.12	3.55	16.10
Mean ± S.D.	1.00 ± 0.01	3.83 ± 0.03	2.33 ± 0.02	10.25 ± 0.04	2.90 ± 0.02	12.96 ± 0.03	3.53 ± 0.02	16.03 ± 0.03

Table 4 (*Continued*)

Parameter	NOR		CAT		ECA		MCA	
	RRT	k'	RRT	k'	RRT	k'	RRT	k'
pH								
2.7	0.98	3.72	2.33	10.22	2.94	13.18	3.56	16.16
3.0	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
3.3	1.02	3.92	2.33	10.22	2.86	12.78	3.51	15.92
Mean ± S.D.	1.00 ± 0.02	3.82 ± 0.02	2.33 ± 0.01	10.22 ± 0.01	2.9 ± 0.03	12.98 ± 0.03	3.53 ± 0.02	16.03 ± 0.02
Sample diluent volume of NaOH (ml)								
0.9	0.98	3.72	2.32	10.18	2.87	12.84	3.49	15.82
1.0	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
1.1	1.03	3.96	2.34	10.28	2.93	13.12	3.57	16.20
Mean ± S.D.	1.00 ± 0.01	3.83 ± 0.03	2.33 ± 0.01	10.23 ± 0.03	2.90 ± 0.02	12.98 ± 0.03	3.53 ± 0.02	16.01 ± 0.04
Temperature (°C)								
37	0.97	3.68	2.38	10.48	3.04	13.66	3.75	17.08
40	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
43	0.99	3.78	2.31	10.14	2.83	12.64	3.45	15.62
Mean ± S.D.	0.99 ± 0.02	3.76 ± 0.02	2.34 ± 0.03	10.28 ± 0.03	2.92 ± 0.02	13.09 ± 0.04	3.58 ± 0.03	16.24 ± 0.04
Flow rate (ml/min)								
0.9	1.02	3.92	2.36	10.38	2.97	13.32	3.56	16.16
1.0	1.00	3.82	2.33	10.22	2.90	12.98	3.53	16.02
1.1	0.98	3.72	2.31	10.14	2.87	12.84	3.51	15.92
Mean ± S.D.	1.00 ± 0.02	3.82 ± 0.02	2.33 ± 0.03	10.25 ± 0.03	2.90 ± 0.02	13.05 ± 0.04	3.53 ± 0.02	16.03 ± 0.03

NOR indicating the NOR is stable in 0.1N NaOH for 24 h.

3.5. Limit of detection and limit of quantification (LOD and LOQ)

The LOD and LOQ values were calculated for NOR and its impurities based on the 3 and 10 times of noise level, respectively, and the values are given in Table 3.

3.6. Robustness

In order to evaluate the robustness of the method the influence of small deliberate variation of analytical parameters on the retention times of NOR and its impurities was studied using two columns of different lots. The parameters selected were mobile phase composition, ionic strength of KH_2PO_4, pH, sample diluent (volume of NaOH), temperature and flow rate. Only one parameter was changed while the others were

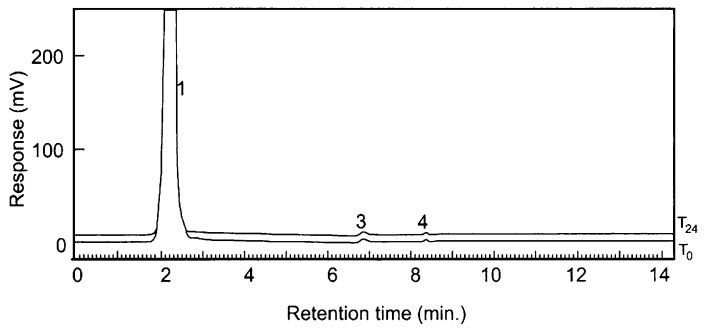

Fig. 6. Typical chromatogram of bulk drug of NOR (200 μg) stored in 0.1N NaOH at ambient conditions for 0 h (T_0) and 24 h (T_{24}).

kept constant. Results are recorded in Table 4. It could be seen from Table 4 that the there is an insignificant change in the relative retention times as well as capacity factors (k') of all the compounds by small deliberate variations.

4. Conclusion

The described isocratic reversed-phase HPLC method for the determination of MCA, ECA and CAT of NOR in bulk drugs and formulations has been evaluated for linearity, precision, accuracy specificity, LOD, LOQ and robustness. The developed HPLC method is suitable not only for separation and determination of process impurities for monitoring of synthetic reactions but also for quality assurance of NOR and related substances.

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