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A STABILITY STUDY OF AQUEOUS SOLUTION OF NORFLOXACIN

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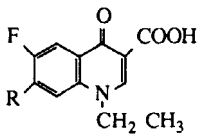
ABSTRACT

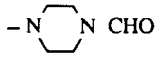
A reversed-phase ion-pair high performance liquid chromatographic assay, which can simultaneously determine norfloxacin and its decomposition products, formyl piperazine and ethylenediamine analogs in aqueous media, has been developed. This assay has been applied to a stability study of norfloxacin in aqueous media. The effects of temperature, pH, oxygen and light on norfloxacin have been investigated using a 2^3X3 factorial design. Results indicate that oxygen, light, temperature and pH have significant effect on the stability of norfloxacin solution. Norfloxacin is most stable at acidic and basic pH, in darkness, in the absence of oxygen and at low temperature.

INTRODUCTION

Norfloxacin (I) is a fluoroquinolone carboxylic acid (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7 [1-piperazinyl]-3-quinoline carboxylic acid) currently in use as a broad spectrum antibiotic[1]. It has been reported that norfloxacin, in its solid state, is



I NORFLOXACIN: R = 

II FORMYL PIPERAZINE: R = 

III ETHYLENE DIAMINE: R = $NH \cdot CH_2 CH_2 - NH_2$

FIGURE 1

Chemical structure of Norfloxacin and its decomposition products.

stable when stored at 40°C and 75% relative humidity and decomposes when exposed to direct sunlight[2]. Two photodecomposition products, formyl piperazine (II) and ethylenediamine (III) analogs (Figure 1) have, so far, been identified.

An acidic solution of norfloxacin when heated to 100°C for 15h has revealed one degradation product i.e., the decarboxylate form and no decomposition was observed in alkaline and hydroalcoholic solutions after 15h of exposure at 100°C[2]. However, no information on the combined effect of oxygen, temperature, light and pH on the stability of norfloxacin solution has been reported. Therefore, a study has been carried out to determine the individual and combined effect of temperature, light, pH and oxygen on the stability of norfloxacin in aqueous media. Since in conventional stability study, interactions among factors are not considered, a factorial experimental design approach[3] has been used for this study. In traditional drug stability studies, the decomposition rate constant of a drug at different storage conditions is usually chosen for analysis[4,5]. However, its determination is tedious

and time consuming. In stability studies of amphotericin B and doxorubicin hydrochloride[6,7], the drug concentration remaining at a fixed time, [(In(In Co - In C)) where C is the concentration of the drug remaining undecomposed at time t and Co is the initial concentration], has been suggested as the dependent variable. Such an approach is therefore adopted in this investigation.

It is also the purpose of the present study to develop a HPLC assay for the measurement of norfloxacin and its degradation products i.e., formyl piperazine and ethylenediamine analogs. The assay developed will be applied to a factorial stability study of norfloxacin in aqueous media.

EXPERIMENTAL

Materials

Norfloxacin and its two analogs i.e., formyl piperazine and ethylenediamine, were supplied by Merck Sharp & Dohme, Rahway, NJ, USA. Orthophosphoric acid and disodium hydrogen phosphate were purchased from BDH, Poole, UK. HPLC grade acetonitrile was obtained from J.T.Baker, Phillipsburg, NJ, USA. Methanol, HPLC grade, was procured from Ajax Chemicals, Sydney, Australia. Sodium lauryl sulphate (99% pure), and tetrabutylammonium bromide were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Water was double distilled and MilliQ® filtered. Glassware was silanized with Aquasil®, obtained from Pierce Chemical Co., Rockford, USA.

Instruments

Chromatography was carried out using a Water Associate M6000A constant flow pump (Milford, MA), and a Schoffel FS970 fluorometer. Sample injection was through a Rheodyne 7125 valve (Cotati, CA, USA) with a 20µl sample loop. The

chromatographic column was stainless steel, 100x2mm ID and slurry packed with 5-µm ODS Hypersil (Shandon Southern Products Ltd. , Cheshire, UK) which had efficiency of over 4000 plates per 10 cm. A glass door oven (Thelco Model 19) was used for the stability study.

Solutions Preparation for the Stability Study

Stock solution of norfloxacin in methanol (100µg/ml) and those of formyl piperazine and ethylenediamine analogs (500µg/ml) were prepared in dichloromethane. Aliquots of the stock solutions were diluted to 10µg/ml with HPLC grade water.

METHODS

Reverse-Phase Ion-Pair High Performance Liquid Chromatography of Norfloxacin and its Decomposition Products for Stability Study

A mobile phase of acetonitrile-water (15:85% v/v) containing 10mM of disodium hydrogen phosphate, 2.5mM of sodium lauryl sulphate (SLS), 10mM of tetrabutylammonium bromide (TBA) adjusted to pH 2 with phosphoric acid, was used. The injection volume was 20µl. A flow rate of 0.5ml/minute was employed. Detection was carried out using a fluorescence detector with an excitation wavelength of 280nm and a 418 emission filter. Peak height was used for quantitation.

Stability Study of Norfloxacin

Table 1 shows the four variables and their levels examined for their effects on the stability of norfloxacin. Temperature, light and oxygen were studied at two levels while the pH effect was studied in acidic, neutral and alkaline conditions, resulting in 2^3x3 storage conditions. These 24 treatments were then randomized for testing.

TABLE 1

Various factors and their levels investigated in the stability study of Norfloxacin

		Level		
	Factor	Low	Medium	High
1.	Temperature	20 ± 4°C (t)	-	70 ± 2°C (T)
2.	Light	darkness (l)	-	250 watts bulb at 50cm (L)
3.	Oxygen	N_2 purged (o)	-	O_2 purged (O)
4.	pH	2.0	7.0	12.0

The upper and lower case letters refer to the high and low level of each factor. For those treatments requiring high light intensity and high temperature, samples were stored in a glass door oven and at the same time were exposed to a 250 watts fluorescent bulb positioned 50cm away from the oven. Samples requiring dark conditions were covered with tin foils. High oxygen level was obtained by purging the sample solutions with 95% oxygen for five minutes and low oxygen levels were ensured by purging with oxygen-free nitrogen for five minutes. The media were adjusted to desired pH with 0.1M sodium hydroxide or 0.1M hydrochloric acid. 9ml of the aliquot solutions of pH 2, 7 and 12 were transferred to silanized glass vials and added with 1ml of norfloxacin solution of 100μg/ml in methanol to give

an initial concentration of 10μg/ml. All the solutions were exposed to appropriate test conditions for 48h. After 48h, samples were removed and cooled immediately by immersing in a ice bath. The remaining drug content was then assayed immediately by reversed-phase ion-pair HPLC.

If necessary, the samples were acidified with 0.1M HCl to facilitate HPLC analysis. Each measurement was made in triplicate and if an individual measurement differed from the others by 5%, further replicate measurements were performed. The data obtained were then analysed by analysis of variance (ANOVA) using the SAS computer package[8].

RESULTS AND DISCUSSION

Reverse-Phase Ion-Pair High Performance Liquid Chromatography of Norfloxacin and its Decomposition Products

^{1}H and ^{13}C NMR investigation of norfloxacin and the two decomposition products in our laboratory has revealed that these compounds are zwitterions[9]. At acidic pH, these compounds will possess a net positive charge. Retention of these compounds can, therefore, be achieved by the addition of anionic pairing ions such as sodium lauryl sulphate or heptane sulphonic acid in the eluent[10]. In this study, the organic modifier concentration, pH and ionic strength of the mobile phase were empirically optimized as previously described[11]. A mobile phase, with 15% v/v acetonitrile content containing 10mM of disodium hydrogen phosphate with pH 2, was selected, as all the analytes were eluted as sharp peaks[12] and also showed maximum fluorescence. Sodium lauryl sulphate was selected as the pairing-ion because of its adsorption characteristics on the C-18 support and its ready availability[13]. Initially, the retention behaviour of the analytes as a function of mobile phase SLS concentration were studied and the results are shown in Figure 2.

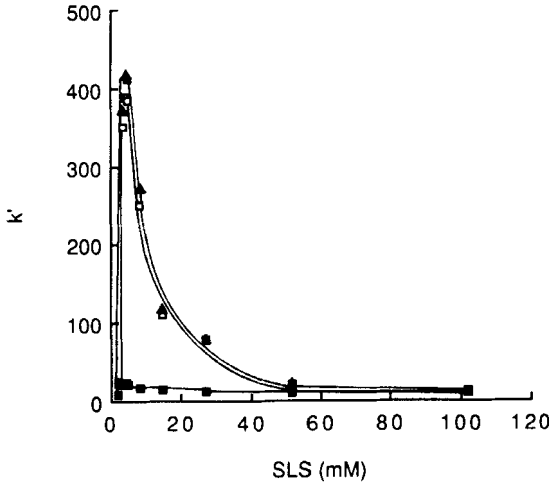


FIGURE 2

Plot showing variation of capacity factor (k') with pairing-ion SLS concentration. Chromatographic conditions: acetonitrile-aqueous buffer (15% v/v) containing 10mM of Na_2HPO4 at pH 2. Peak identification: (□) Norfloxacin; (■) Formyl piperazine analog and (▲) Ethylenediamine analog.

The capacity factors (k') for all the analytes passed through the predicted maxima[14,15], as suggested by ion-exchange desolvation mechanism[13]. The separation among the analytes was achieved at 2.5mM SLS with retention times of norfloxacin, formyl piperazine and ethylenediamine analogs of 190, 8 and 205 minutes respectively. In order to reduce the retention time of the analytes while retaining the separation among them, organic counter ion, TBA, was added to the mobile phase[16,17]. The retention of the analytes at 2.5mM SLS, 15%v/v acetonitrile-buffer and at pH 2, as a function of mobile phase TBA concentration is shown in Figure 3. The retention of all the compounds was found to decrease with increase in the mobile phase TBA concentration. Acceptable retention and separation among the analytes were obtained with a mobile phase of 15% v/v acetonitrile-buffer, 2.5mM SLS, 10mM TBA and at pH 2. Figure 4 illustrates a

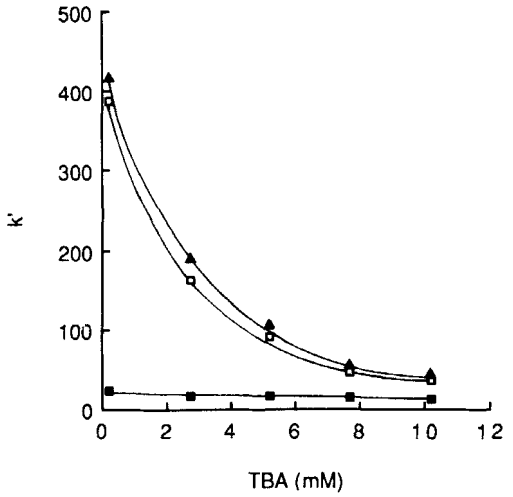


FIGURE 3

Plot showing variation of k' with mobile phase TBA concentration. Peaks as given in figure 2 and chromatographic conditions: acetonitrile-aqueous buffer (15% v/v) containing 10mM Na_2HPO4 and 2.5mM SLS at pH 2.

chromatogram using the above selected mobile phase with retention time of 14.2, 3 and 16 minutes for I, II and III respectively and this mobile phase was used for subsequent stability studies.

Calibration curves of peak height (20µl injected) versus concentration were prepared for all three compounds and were found to be linear in the concentration range given in Table 2. The coefficients of determination greater than 0.99 were observed for all the analytes. Both within day and between day coefficients of variation were less than 5%. Using signal to noise ratio of 3 as a criterion, the detection limit for norfloxacin, ethylenediamine and formyl piperazine analogs were about 25,10 and 50ng/ml respectively.

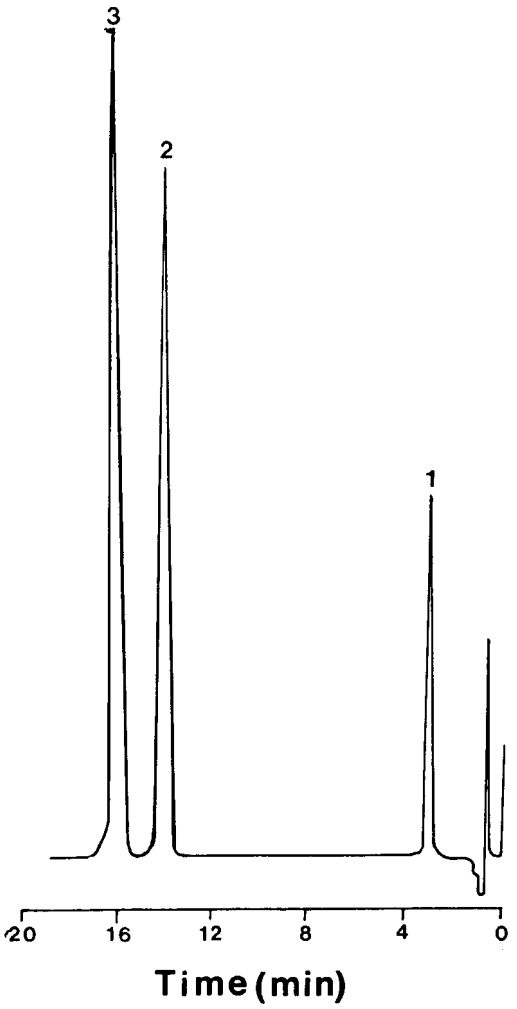


FIGURE 4

Representative chromatogram showing separation of the three compounds. Peaks: (I) Norfloxacin; (II) Formyl piperazine analog and (III) Ethylenediamine analog. Chromatographic conditions: acetonitrile-aqueous buffer (15% v/v) containing 10mM Na_2HPO4, 2.5mM SLS, 10mM TBA at pH 2.

TABLE 2

Regression Data for the Calibration Curves for (I) Norfloxacin ; (II) Formyl piperazine analog and (III) Ethylenediamine analog

Drug	Calibration range (μg/ml)	Equation[a]	r^2	n[b,c]
I	0.1 - 5.0	Y= 0.1064 + 3.8942X	1	7
II	0.1 - 10.0	Y= 0.0034 + 0.8706X	1	9
III	0.1 - 2.5	Y= 0.0284 + 6.8078X	1	6

[a]X = concentration of I, II and III

Y = peak height in cm

b = number of concentration points on each curve

c = six samples were tested at each concentration

Factorial Design and the Stability Study of Norfloxacin

The pH of all the treatments, measured after the stability study, was found to vary not more than 0.3 pH units. The concentrations of norfloxacin remaining after accelerated stability procedure are given in Table 3. Initial attempts to fit the data using zero order transformation were not successful. Examination of the residuals indicated that log-log transformation of the concentration (In (InCo-InC)) as the dependent variable is appropriate in analysing the data. Hence, a first order decomposition can be assumed under all storage conditions examined in this study. Since the experiments were not repeated, the 4-way interaction was used as the

TABLE 3
Concentration of Norfloxacin Remaining after Accelerated Stability Conditions[b]

	Treatment Combination	Concentration remaining[c] pH 2.0	pH 7.0	pH 12.0
1.	tol	9.76	9.19	9.70
2.	toL	9.55	9.12	9.68
3.	tOl	9.50	8.91	9.65
4.	tOL	9.43	8.73	9.18
5.	Tol	9.65	8.90	9.51
6.	TOl	9.64	8.33	9.56
7.	ToL	9.45	8.79	9.46
8.	TOL	9.15	7.15	9.10

a Initial concentration of Norfloxacin = 9.98μg/ml
b Mean of four sets of data
c Data obtained after storage of drug samples for 48h under the specified conditions

error term in the initial analysis. All the interaction terms were found to be insignificant at 5% level. To increase the sensitivity of the analysis, all the interaction sums of square were pooled into the error sum of squares in the second analysis. The effect of all the four factors were found to be highly significant (P values in bracket); pH (0.0001), oxygen (0.0002), light (0.0003) and temperature (0.0016). In order to evaluate the effect of different pH on the stability of norfloxacin, Tukey's Studentized Test[18] was then performed on the three levels of pH. The mean value at pH 2 and 12 were not significantly different at the 5% level

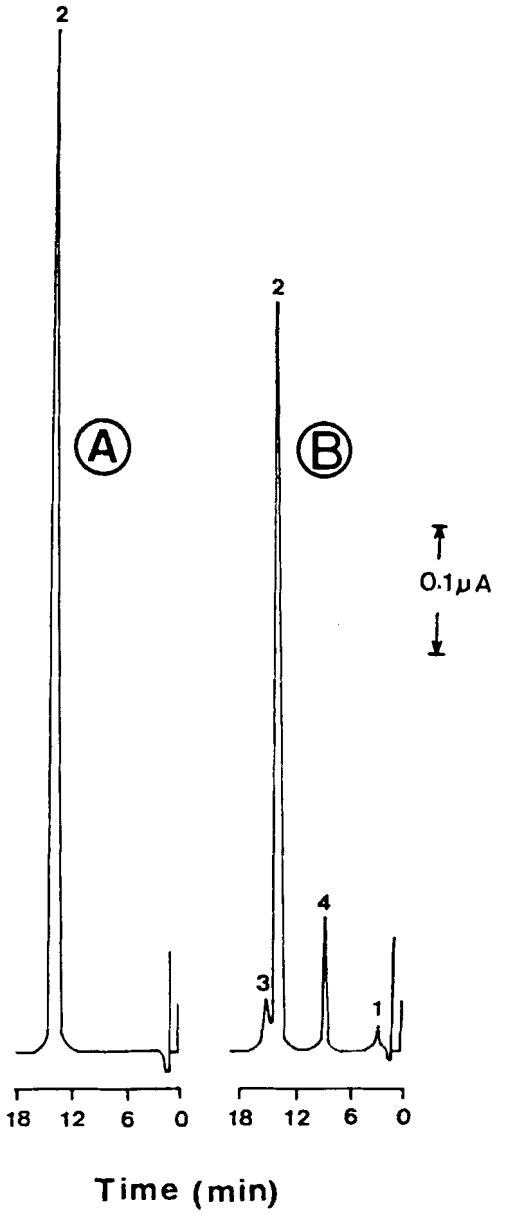


FIGURE 5

Representative chromatogram of Norfloxacin: (A) in aqueous solution at pH 7 before subjecting to accelerated conditions and (B) in aqueous solution at pH 7 after 48h of storage at 70°C in presence of light and oxygen. Peak identification: (I) Norfloxacin; (II) Formyl piperazine analog; (III) Ethylenediamine analog and (IV) unidentified decomposition peak.

while a significant difference was observed at neutral pH 7. The results presented in Table 3 demonstrate that when subjected to harsh conditions of temperature, oxygen and light, norfloxacin decomposes upto 30% under neutral conditions. However, at acidic and basic pH, it remains stable irrespective to any severe situation. A norfloxacin sample (9.98 μg/ml as the initial concentration) of pH 7 following 48h of storage at 70°C in presence of light and oxygen was found to have 7.15μg/ml of norfloxacin, 0.45 μg/ml of formyl piperazine, 0.23μg/ml of ethylenediamine analog and a fluorescent compound having retention time of 9.0 minutes (Figure 5).

Chromatogram of norfloxacin solution subjected to higher temperature levels in all cases showed an unknown peak other than II and III and this may be the decarboxylated form reported earlier[2]. In a separate experiment, an acidic solution of norfloxacin at pH 2.0, when heated for 15h in a boiling water bath in absence of light and oxygen, showed a small peak with retention time same as that of unknown peak. The results indicate that simultaneous presence of oxygen and light has a catalytic effect on the decomposition of norfloxacin and the maximum decomposition occurs at high temperature in the presence of light and oxygen. Norfloxacin is therefore most stable at acidic and basic pH, in darkness, in the absence of oxygen and at low temperature.

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