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High-performance liquid chromatographic method for the determination of norfloxacin glutamate and glucuronate in solid and liquid dosage forms and its application to stability testing

CHEN CUIPING,*† LIU XINGRONG‡ and WU RUJIN†

†*Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing, 210009, P.R. China*
‡*Jiangsu Research Institute of Sports Science, Nanjing, 210014, P.R. China*

Abstract: A simple, precise, stability-indicating reversed-phase high-performance liquid chromatographic method for norfloxacin glutamate and norfloxacin glucuronate in liquid and solid dosage forms is described. Chloronitrodiazepine was used as the internal standard. The eluent used with a C18 bonded phase column was methanol–water–diethylamine (50:50:0.4, v/v/v) (pH* 5.5). The effects of the eluent pH*, the ratio of methanol to water, and the quantity of diethylamine on the retention times of the sample and internal standard were investigated. The method showed good linearity in the range 1–45 μg ml^{-1} for norfloxacin. Solid samples were ground, dissolved in the eluent, filtered, and then determined by this method. Liquid samples were dissolved in the same solvent. The average recoveries of norfloxacin glutamate and norfloxacin glucuronate in their simulated preparations were 99.5% for solid products and 99.8% for liquid products. The method was applied to the study of the thermal stability of the drugs by following the degradation of norfloxacin glutamate and glucuronate in the four products in accelerated tests at 37–80°C for up to 3 months. Shelf-lives at 25°C of the four products were predicted from the results assuming zero- and first-order kinetics of decomposition, and were at least 1.5 years for liquid products and 2 years for solid products.

Keywords: *High-performance liquid chromatography; norfloxacin glutamate; norfloxacin glucuronate; stability testing.*

Introduction

Norfloxacin (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazin-1-ylguinoline-3-carboxylic acid) has been widely used as a broad-spectrum anti-bacterial substance and is one of the drugs of first choice for the treatment of, for instance, complicated urinary tract infections and enteric infections [1]. Owing to its poor solubility in common solvents, two salts of norfloxacin were prepared, norfloxacin glutamate and norfloxacin glucuronate. Furthermore injection and tablet forms of these salts were developed and showed improved bioavailability.

Although several HPLC methods have been reported for the determination of norfloxacin [2–5], all need a counter-ion in the mobile phase or a fluorescence detector. In the present paper, a new HPLC assay with UV detection is reported. This analytical method is simpler and is specific for the evaluation of the stability of the two salts of norfloxacin in formulated products. The method has been used for the thermal stability study of the two salts of norfloxacin and the shelf-lives of their products have been predicted.

Materials and Methods

Instrumentation

Chromatographic analyses were carried out using a Hewlett–Packard 1050 series liquid chromatograph with a multiple-wavelength photodiode-array detector. The detector was set at 278 nm and the signal recorded on a Hewlett–Packard 3396A integrator.

Chemicals and reagents

Norfloxacin and its two salt standards and the internal standard, chloronitrodiazepine, were kindly provided by the Department of Pharmaceutics of the authors' University. All reagents were of analytical reagent grade and double-distilled water was used.

HPLC

A 100 × 4.6 mm i.d. column was packed

* Author to whom correspondence should be addressed.

with 5-μm Hypersil ODS. The separation of sample and internal standard from impurities was achieved isocratically using methanol–water–diethylamine (50:50:0.4, v/v/v) as the eluent at a flow rate of 1 ml min^{-1}. The eluent pH* was adjusted to 5.5 by adding concentrated phosphoric acid. The eluent was filtered (pore size 0.6 μm) before use and then degassed by helium throughout the experiment. The injection volume was 20 μl. The assays were performed at ambient temperature.

Optimization of the HPLC conditions. The optimization experiment was conducted in order to establish a sensitive and simple method for the analysis of the two products.

The effects of the ratio of methanol of water, the eluent pH* and the ratio of organic amine in the mobile phase on the resolution, retention times and peak shapes of both sample and internal standard were studied.

Standards and sample preparation. The norfloxacin standard (100 mg) was weighed accurately and transferred with the aid of approximately 20 ml of 0.85% phosphoric acid solution into a 100-ml volumetric flask. The mixture was thoroughly shaken until all the ingredients had dissolved and then diluted to volume with double-distilled water; this served as the stock solution. A 1 ml volume of the stock solution was placed in another 100-ml volumetric flask and then diluted with the mobile phase to volume; the concentration of norfloxacin was about 10 μg ml^{-1}. The internal standard solution was prepared in the same way at a concentration of about 20 μg ml^{-1} in the same way but methanol was used instead of double-distilled water as the solvent in the first step. Aliquots of an appropriate volume of the norfloxacin and internal standard solutions were taken to make a series of mixed solutions in the range 5.0–9.00 μg ml^{-1} for norfloxacin and 2.0–10.00 μg ml^{-1} for the internal standard.

For the solid samples, 20 tablets (each containing 125 mg of norfloxacin) were accurately weighed and ground to a powder. An appropriate amount of the powder was weighed accurately. The powder was dissolved in water with ultrasonication; the solution was filitered and an appropriate amount of internal standard added. The solution was diluted further with the eluent so that the concentrations of norfloxacin and the internal standard in the solution were near 7 and 8 μg ml^{-1}, respectively. For the liquid samples, to an aliquot of appropriate volume was added an appropriate amount of the internal standard; the solution was then diluted with the eluent to a concentration of 7 μg ml^{-1} for norfloxacin and 8 μg ml^{-1} for the internal standard.

Test for peak purity. Peak purity was tested to determine the reliability of the method for use in stability tests. The ratios of absorbance of the samples and the internal standard at 278 and 270 nm against the corresponding elution time over the areas of the peaks were plotted, using a Hewlett–Packard 1050 UV photodiode-array detector.

Stability testing [6]

This test was required to determine the expiry date of drug preparations. Usually a study of the stability of drug substances towards degradation by factors such as heat, oxygen, light or humidity is carried out under ambient and/or accelerated conditions.

In the present work, accelerated tests were conducted and the results were extrapolated to ambient conditions. Using straightforward chemical kinetics, the order of the reaction must be determined or assumed. For a zero-order reaction, the relationship between the concentration of the drug [C] at time t relative to its initial concentration $[C]_0$ is given by equation (1):

$$[C] = [C]_0 - k_0 t, \qquad (1)$$

where k_0 is the rate constant. Thus a plot of [C] versus t should be linear with a negative slope.

For a first-order reaction, the relationship is given by equation (2):

$$\ln[C] = \ln[C]_0 - k_1 t. \qquad (2)$$

Thus a plot of ln[C] versus t should be linear. For the thermal stability testing, the relationship between the rate constant, k, and temperature is given by the Arrhenius equation:

$$\ln k = \ln A - Ea/RT, \qquad (3)$$

where A is a constant, Ea is the energy of activation of the degradation reaction, T is the absolute temperature, and R is the gas constant, 1.987 cal mol^{-1} K^{-1}. Therefore, the rate

constant at ambient temperature (25°C) can be calculated from equation (3).

In this experiment, the four products were subjected to thermal stability tests in which they were stored for up to 3 months at 37, 47 and 57°C for solid samples and 40, 60 and 80°C for liquid samples in a constant temperature oven capable of temperature control to ±1.0°C.

Results and Discussion

Optimization of chromatographic conditions

A typical chromatogram of norfloxacin and the internal standard is shown in Fig. 1(A); the retention times were 3.2 min (norfloxacin) and 6.9 min (chloronitrodiazepine). The chromatogram clearly demonstrated that the separation was complete as well as rapid. The limit of detection of the assay without interference was 0.01 μg ml^{-1} for norfloxacin.

The effects of the ratio of methanol to water, eluent pH* and diethylamine volume in the mobile phase on the retention time are shown in Fig. 2.

The results showed that the eluent pH* and the organic amine had a great influence on the retention time (t_R) of norfloxacin. Its t_R increased as the pH* increased and the peak tailed gradually at the same time; its t_R decreased as the organic amine volume increased and the peak shape was found to be greatly improved. On the other hand both eluent pH* and organic amine volume had no striking effect on the t_R of the internal standard; its t_R remained nearly constant as the two factors were changed. The ratio of methanol to water had the same influence on the sample

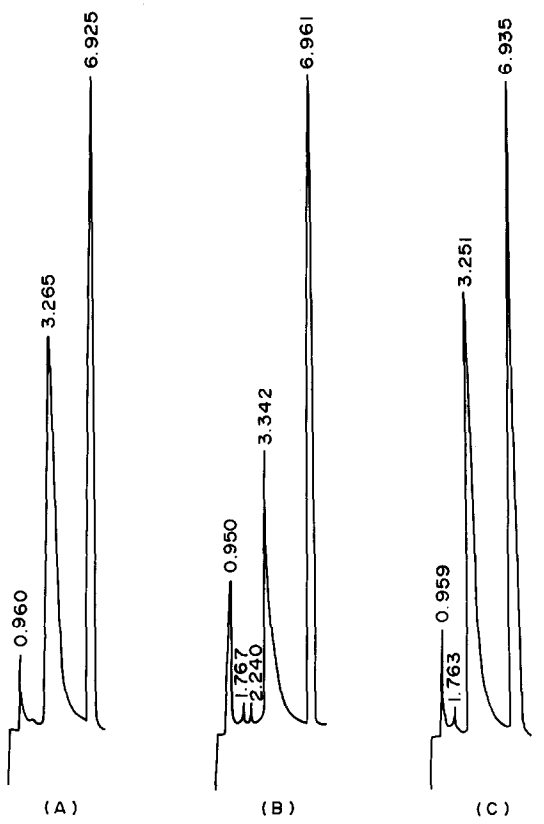

Figure 1
Chromatograms of norfloxacin and the internal standard (A), norfloxacin glucuronate in solid (B) and liquid (C) forms with the internal standard. Retention time was about 3.2 min for norfloxacin and 6.9 min for the internal standard.

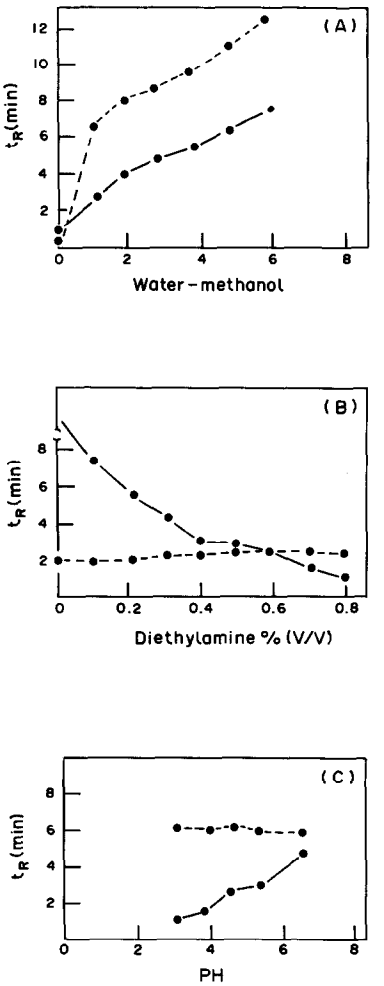

Figure 2
Effects of the water–methanol ratio (A), diethylamine content (B) and the pH* of the mobile phase (C) on the retention time (t_R) of norfloxacin (——) and chloronitrodiazepine (----), mobile phase was as follows: (A) 0.3% diethylamine, pH* 6; (B) methanol–water = 60:40, v/v, pH* 6; (C) methanol–water = 60:40, v/v, 0.3% diethylamine. Other conditions were the same as those mentioned in the text.

and the standard but it was necessary for baseline separation that the ratio of methanol to water should be less than 60:40.

Linearity

Norfloxacin standard instead of its salts standards was used in the linearity test because it had been confirmed in experiments that they showed identical chromatographic behaviour under the proposed HPLC conditions. The ratios of the peak areas of the norfloxacin standards to those of the internal standards (As/Ais) versus the corresponding concentration ratios (Cs/Cis) were calculated by linear regression analysis. Five standard solutions were injected. The calibration plot was linear within the range 1–45 $\mu g\ ml^{-1}$ for norfloxacin; the correlation coefficient was 0.9999 ($n = 6$).

Recovery study

Simulated solid and liquid products were prepared by adding appropriate amounts of norfloxacin glutamic acid and glucuronic acid to placebos made according to the corresponding formulae and were determined by the proposed methods. The recoveries averaged 99.5% for the solid forms and 99.8% for the liquid forms; the relative standard deviations were less than 1.5% ($n = 5$).

Stability tests

From the stability tests, two results were observed. First, the ratio chromatograms of the samples and the internal standard in all cases were square; this result meant that only one compound was eluted within a peak area and that there was no interference by possible degradation products or other impurities. An example of a ratio chromatogram is given in Fig. 3.

Second, although impurity peaks appeared

Figure 3
Ratio chromatogram of a liquid sample; the wavelengths used were 278 and 270 nm.

in the chromatograms of the two norfloxacin salts in solid and liquid preparations (Fig. 1(B), (C)), the HPLC assay results in Table 1 showed that the extent of decomposition of the two newly developed products was small; the changes in their concentrations were less than 7% over the period investigated, and it was impossible to identify the reaction order from the experimental plots. HPLC assay results at each temperature were plotted both as a percentage of initial concentration of norfloxacin versus time (days), corresponding to zero-order kinetics, and as ln (% of initial concentration) versus time (days), corresponding to first-order kinetics. The rate constants at the three temperatures were calculated from the slopes of these lines. Then, a plot of $\ln k$ versus $1/T$ allowed extrapolation of the rate constant at 25°C. Thus the predicted shelf-life

Table 1
The ratio (%) of the concentrations of norfloxacin glutamate and norfloxacin glucuronate to the initial concentrations under different storage conditions

Temp. (°C)	Norfloxacin glutamate Time (days)			Norfloxacin glucuronate Time (days)		
	30	60	90	30	60	90
37	99.62	98.35	98.27	99.53	98.46	98.20
47	98.62	97.82	96.23	98.63	97.82	96.29
57	98.10	97.39	95.09	98.30	97.34	95.55
40	99.41	98.20	97.81	99.01	98.47	97.35
60	98.67	96.70	95.58	98.70	97.01	95.87
80	98.08	94.96	93.82	98.49	95.39	94.54

Table 2
Shelf-lives of norfloxacin glutamate and norfloxacin glucuronate in solid and liquid preparations, predicted from results of accelerated tests assuming zero-order and first-order kinetics

Sample		Shelf-lives (days)	
		Zero-order kinetics	First-order kinetics
Norfloxacin glutamate	solid	721	739
	liquid	545	567
Norfloxacin glucuronate	solid	712	740
	liquid	540	581

at 25°C was: for zero-order kinetics, from equation (1): $t_{10\%} = (100-90)/k_0$; and for first-order kinetics, from equation (2), $t_{10\%} = (\ln 100 - \ln 90)\ k_1$. Results are shown in Table 2. The predicted shelf-lives of the liquid forms at 25°C were more than 1.5 years and those of the solid forms were more than 2 years.

Conclusions

A reversed-phase high-performance liquid chromatographic method for the determination of norfloxacin has been established. The method has been used to determine two newly developed norfloxacin salts, norfloxacin glutamate and norfloxacin glucuronate, in solid and liquid preparations and has also been applied to stability tests of their products.

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