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Short communication

Rapid and economical high-performance liquid chromatographic method for the determination of norfloxacin in serum using a microparticulate C_{18} guard cartridge

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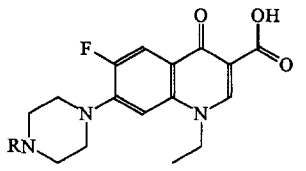
Abstract

A rapid and economical high-performance liquid chromatographic assay is described for norfloxacin in serum. Samples (100 μl) containing N-ethylnorfloxacin as the internal standard were extracted into 1 ml of chloroform. Chromatography was performed at 30°C on a 40 × 3.2 mm I.D. C_{18} guard cartridge (3 μm spherical particles) using a mobile phase of 11% (v/v) acetonitrile in 0.01 *M* phosphate buffer (pH 2.5) containing 0.001 *M* triethylamine, and pumped at 1 ml/min. Detection was at 279 nm. The retention times of norfloxacin and internal standard were 1.9 and 2.9 min, respectively. Calibration curves were linear ($r > 0.999$) from 0.1 mg/l to at least 2.0 mg/l. Within-day and between-day precision (C.V.) were 8.6% or less, and accuracy was 5.3% or less. Absolute assay recovery of norfloxacin was over 70%.

Keywords: Norfloxacin

1. Introduction

Norfloxacin (Fig. 1) was the first member of the fluoroquinolone family of antimicrobial agents available for clinical use in the United States and is now widely used elsewhere. It has proved effective against Gram-positive and Gram-negative pathogens and is particularly useful for treating difficult urinary tract infections [1]. A number of methods for determining norfloxacin concentrations in human serum or plasma have been published, including complicated extraction procedures [2,3] and column switching [4]. Some have involved protein precipitation which can have a detrimental effect on the life



R = H, NORFLOXACIN
R = CH_2CH_3, N-ETHYLNORFLOXACIN

Fig. 1. Chemical structure of norfloxacin and N-ethylnorfloxacin (internal standard).

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and performance of expensive analytical columns [5–8], while another used ion-exchange chromatography [9] which is relatively inefficient and suffers from increased deactivation compared with reversed-phase methods.

We required a simple, accurate and precise method suitable for the analysis of large numbers of samples in pharmacokinetic studies. Besides fulfilling these requirements, the procedure reported here is extremely rapid with a total chromatographic run-time of less than 5 min. It is also cost-effective due to the use of a relatively inexpensive guard cartridge as the analytical column which requires considerably lower concentrations of expensive polar modifiers in the mobile phase than other methods.

2. Experimental

2.1. Reagents and materials

Norfloxacin (Sigma, St Louis, MO, USA) was used as received. N-ethylnorfloxacin, the internal standard, was synthesised from norfloxacin by the procedure of Koga et al. [10]. Acetonitrile, methanol, and chloroform were HPLC grade and purchased from EM Science (Gibbstown, NJ, USA). Other chemicals were AR grade. Fresh, glass-distilled water was used in the preparation of buffers and the mobile phase.

2.2. Instrumentation

Analyses were performed on a modular system consisting of a Model M45 pump, a Model 712 WISP injector, a Model 484 variable wavelength detector, a Temperature Control System (Millipore, Waters Chromatography Division, Milford, MA, USA), and a chart recorder (Omniscribe, Houston Instruments, Austin, TX, USA).

2.3. Standards and controls

The norfloxacin used to prepare standards and controls was weighed on separate balances to minimise systematic bias. Stock solutions of norfloxacin in methanol (40 mg/ml) and N-ethylnorfloxacin in chloroform (2.6 mg/l) were prepared. An appropriate volume of norfloxacin stock was dispensed into glass tubes, the solvent was evaporated and the norfloxacin residue redissolved in drug-free serum to yield standards of 0.1, 0.2, 0.4, 0.8, 1.4, 2.0 mg/l. Similarly, a replicate stock solution was prepared to give seeded controls of 0.15, 0.6, and 1.7 mg/l in serum. Standards and controls were dispensed in 100-μl aliquots and stored at −70°C.

2.4. Sample preparation

Serum samples (100 μl) were pipetted into 1.5 ml conical polypropylene tubes (Eppendorf) and extracted into 1.0 ml of chloroform containing 2.6 μg of N-ethylnorfloxacin by vortex agitation. The tubes were centrifuged at 11 000–12 000 *g* for 1 min, and the clear organic phase was decanted into glass tubes and evaporated under an airstream at 60°C. The residue was reconstituted in 200 μl of mobile phase, of which 25–50 μl was injected into the chromatograph.

2.5. Chromatography

The mobile phase of 11% (v/v) acetonitrile in a pH 2.5 buffer of 0.01 *M* sodium dihydrogen orthophosphate and 0.001 *M* triethylamine was filtered (0.45 μm pore size) and degassed under reduced pressure before use. The flow-rate was 1.0 ml/min. Separations were performed on a 40 × 3.2 mm I.D. guard cartridge containing 3-μm spherical particles (RP-18 Spheri-3; Applied Biosystems, San Jose, CA, USA). The column temperature module was set to 30°C to avoid temperature-dependent fluctuations in retention time. Detection was at 279 nm, the experimentally determined spectral maximum for norfloxacin and N-ethylnorfloxacin in the mobile phase, and the chart recorder sensitivity was set at 0.01 AUFS (absorbance units full scale deflection).

2.6. Quantitation, precision, accuracy

Calibration plots were constructed from least-squares regression analysis of the norfloxacin to N-ethylnorfloxacin peak-height ratios. Unknowns

were calculated by inverse prediction. Standards, controls, and unknown samples were processed identically. Within-day precision and accuracy were estimated by analysing the three seeded controls six times each during one day in no fixed order, while between-day precision and accuracy was determined by analysis of the three controls on six separate days using a new calibration each day.

2.7. Recovery

Absolute percentage recovery was estimated by analysing nine standards and controls of norfloxacin in serum and comparing the peak heights obtained with that obtained from the direct injection of known amounts of norfloxacin in phosphate buffer.

3. Results and discussion

The norfloxacin derivative, N-ethylnorfloxacin, was synthesised by the alkylation of the external piperazinyl nitrogen of norfloxacin as described previously [10]. This close structural analog of norfloxacin was an excellent internal standard because it has similar UV characteristics and chromatographic retention to norfloxacin. Two other fluoroquinolone antimicrobials, ciprofloxacin and pefloxacin, coeluted with norfloxacin and, therefore, this assay method has the potential for measuring these agents with little or no modification. Norfloxacin and N-ethylnorfloxacin had retention times of 1.9 and 2.9 min, respectively, and were eluted in an area of the chromatogram free from interfering endogenous peaks. Fig. 2 shows the analysis of drug-free serum, an extracted norfloxacin standard containing 0.8 mg/l, and a serum sample containing 0.61 mg/l drawn 2.5 h after a healthy male volunteer (age 26 yr, weight 70 kg) took a 400-mg tablet of norfloxacin (Noroxin, Merck Sharp and Dohme) with 250 ml water.

Approximately one-third of a norfloxacin dose is metabolised in the liver to the more polar metabolites including the oxo- and 7-ethylenediammine derivatives. The specificity of the assay in the presence of various norfloxacin metabolites could not be ascertained since pure reference materials were unobtainable. However, others have shown that concentrations of these compounds in serum is either extremely low or undetectable even after very high doses of 1600 mg [11].

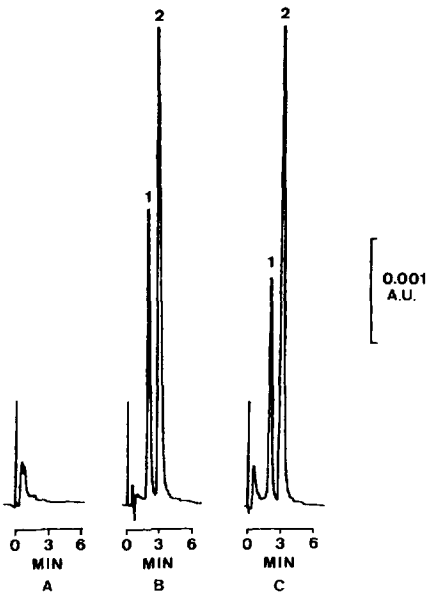

Fig. 2. Chromatogram of: (A) drug-free human serum; (B) extracted norfloxacin standard, 0.8 mg/l; (C) serum containing 0.61 mg/l taken 2.5 h after a healthy subject took a 400-mg oral dose of norfloxacin. Peaks: 1 = norfloxacin; 2 = N-ethylnorfloxacin (internal standard).

Calibrations were linear ($r > 0.999$) from 0.1 mg/l to at least 2.0 mg/l. Mean (±S.D.) calibration plots ($n = 8$) gave the following equation to a straight line: $y = 1.10(\pm 0.13)x + 0.016(\pm 0.015)$, where y is the peak-height ratio and x is the norfloxacin serum concentration. The minimum detectable concentration (three times baseline noise) was 0.02 mg/l. While greater sensitivity has been reported elsewhere with fluorescence detection [3,5,8] the present method can measure norfloxacin down to 5–10% of the average peak concentrations (1.5–2.0 mg/l) achieved in serum after a 400-mg oral dose. This spans 4–5 terminal elimination half-lives which is acceptable for norfloxacin pharmacokinetic applications. Furthermore, the more specialised

fluorescence detectors have a higher initial capital outlay and more expensive xenon lamps than UV detectors. Within-day precision, between-day precision, and accuracy all were 8.6% (C.V.), or less, in the range of 0.15 mg/l to 1.7 mg/l (Table 1), the concentration range typically encountered after administration of normal clinical doses [12]. The mean (±S.D.) ($n = 9$) assay recovery between 0.1 and 2.0 mg/l was 70.3 (±4.9)%.

Compared with standard reversed-phase analytical columns the use of the less expensive microparticulate guard cartridge permitted adequate resolution of the two peaks of interest while providing increased cost-saving measures since only small amounts of a polar modifier (e.g. acetonitrile) are required in the mobile phase. Furthermore, it was found that the amount of acetonitrile could be progressively decreased from 11% with new cartridges or, after cleaning, to about 4–5% (v/v) with continued use over time. Cleaning was achieved periodically by flushing with methanol with the addition of dimethylsulphoxide, if necessary. Typically, the life of the guard cartridge was greater than 1400 injections, provided standard care and maintenance practices were observed. Sustained deviations from linearity at the high end of the calibration range and excessive broadening of analyte peaks indicated the end of useful cartridge life.

Table 1
Precision and accuracy of HPLC norfloxacin analysis in serum

Concentration (mg/l)			C.V. (%)	Accuracy[a] (%)
Target	Found	S.D.		
Within day (n = 6)				
0.15	0.146	0.011	7.8	2.7
0.6	0.593	0.051	8.6	1.2
1.7	1.623	0.104	6.4	4.5
Between day (n = 6)				
0.15	0.158	0.012	7.5	5.3
0.6	0.613	0.026	4.3	2.2
1.7	1.649	0.138	8.3	3.0

[a] [Absolute (target − found)/target] × 100
S.D. = standard deviation; C.V. = coefficient of variation.

In conclusion, we have described a simple, rapid, sensitive and precise means of analysing norfloxacin in serum with distinct cost-saving benefits. This method further extends and supports previous work by ourselves [13] and others [14] that reversed-phase HPLC guard cartridges provide a useful and economical means for the analysis of a variety of drugs used in common clinical practice.

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