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Short communication

Determination of norfloxacin in human plasma and urine by high-performance liquid chromatography and fluorescence detection

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Abstract

A method for the determination of norfloxacin in human plasma and urine is described. Plasma samples were deproteinized using acetonitrile. The supernatant was analysed by C_{18} HPLC. Fluorescence detection at an excitation wavelength of 300 nm and an emission wavelength of 450 nm was utilized. The assay was validated in the concentration range of 31 to 2507 ng/ml when 0.5-ml aliquots of plasma were handled. The intra-day precision of the spiked quality control samples ranged from ±0.37 to ±4.14% in plasma (concentration range: 70.3–2109.2 ng/ml) and from ±0.51 to ±1.56% in urine (concentration range: 7.5–299.4 μg/ml). The intra-day accuracy obtained for norfloxacin in the quality control samples ranged from −5.18% to −9.47% in plasma and from −10.56% to −5.91% in urine. The assay has been used to support human pharmacokinetic studies.

Keywords: Norfloxacin; Antibiotics

1. Introduction

Norfloxacin is a fluorinated 4-quinolone antimicrobial agent structurally related to nalidixic acid, but with a wider antibacterial spectrum and greater activity [1]. About 30% [1] of a dose is excreted unchanged in the urine within 24 h thus producing high urinary concentrations. It is therefore used in the treatment of urinary tract infections in usual doses of 400 mg twice daily. Consequently, it was important to develop a assay also suitable for analysis of norfloxacin in urine.

Previous works dealing with the determination in plasma, tissue and urine were done by liquid–solid extraction [2,3], simple dilution [4], protein precipitation [5] or liquid–liquid extraction [6,7] in connection with DC [3] or HPLC with UV [5–7] or fluorescence detection [2,4].

There were other methods published, however, where chromatographic separation was not used, but only the fluorescence behaviour of norfloxacin [8,9].

Determination without chromatographic separation may suffer from selectivity concerning endogenous substances or metabolites. Considering the calibration range, some published methods are not sensitive enough [4–7] for the determination of norfloxacin following single oral doses. One group [3] is using thin-layer chromatography which is not so often used in analytical laboratories for pharmacokinetic studies. Another group [2] published a very sensitive method by using a specific, self-prepared precolumn.

The liquid–liquid extraction of norfloxacin as an amphoteric molecule is extremely pH dependent. The present paper used protein precipitation with acetonitrile to avoid extraction of norfloxacin and

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reversed-phase HPLC in connection with fluorescence detection. Therefore, the method presented uses a simple and fast sample preparation with a low limit of determination.

2. Experimental

2.1. Materials

Norfloxacin was obtained from Biochemie (Kundl, Austria). Acetonitrile and methanol (HPLC grade) were obtained from Rathburn (Walkerburn, UK). All other reagents were of ACS grade and were used as received (E. Merck, Darmstadt, Germany).

2.2. Instrumentation

The HPLC system consisted of a HP 1090M liquid chromatograph (Hewlett–Packard, Waldbronn, Germany) and a fluorescence detector F1050 (Merck–Hitachi, Darmstadt, Germany). The analog output from the detector was connected to a PE-Nelson 2600 data system (Perkin–Elmer, USA) via a PE-Nelson Series 900 analog-to-digital interface.

2.3. Chromatographic conditions

The mobile phase consisted of 30% methanol and 70% 0.1 *M* perchloric acid and 0.02 *M* triethylamine in water (v/v) and was degassed using helium. The pump delivered the mobile phase at a flow-rate of 1.0 ml/min (pressure approx. 2000 p.s.i.; 1 p.s.i.= 6894.76 Pa). The use of methanol resulted in a better peak shape than acetonitrile. The relatively high molarity of perchloric acid was necessary for a satisfying peak shape; otherwise strong tailing resulted by using lower concentrations or other types of acids. The analytical column was a 80×4.0 mm I.D. stainless steel column packed with 3-μm Nucleosil $3C_{18}$ bonded silica (SRD-Pannosch, Vienna, Austria). The column was operated at a temperature of 40°C.

The sample injection volume was 20 μl. The fluorescence detector was set at an excitation wavelength of 300 nm and an emission wavelength of 450 nm (time constant 1 s).

2.4. Preparation of standards

A 400-μg/ml solution of norfloxacin was prepared by weighing 20 mg into a 50-ml volumetric flask and then dissolving the substance in methanol.

Plasma standards in the range of 30–2400 ng/ml were prepared by spiking blank human plasma with this solution (400 μg/ml).

2.5. Plasma extraction procedure

Plasma samples were thawed and mixed. An aliquot of 0.5 ml was transferred to a glass reaction tube and 0.5 ml of acetonitrile was added. The sample was vortex-mixed vigorously for 20 s and then centrifuged for 2 min at 4000 rpm (>2000 *g*). A 250-μl volume of the supernatant was transferred into a glass autosampler vial and mixed with 500 μl of a solution of 0.1 *M* perchloric acid and 0.02 *M* triethylamine in water. A 20-μl volume was injected into the HPLC system.

3. Results and discussion

3.1. Linearity

For evaluation of the calibration graph, a weighted linear regression (1/concentration) was performed with nominal concentrations of calibration standards against measured peak areas. The linearity of the assay could be shown over a concentration range of 31–2507 ng/ml of plasma. The coefficient of correlation (R^2) was at least 0.999. The precision of the individual calibration points were between ±0.22 and ±2.75%. The mean accuracy of the different calibration levels was between −1.87 and 1.34%. The lowest calibration standard used in the validation calibration curve yielded a concentration of 31.0 ng/ml of plasma. This was set as the limit of quantification that can be measured with certain accuracy (concentration, calculated: 31.0 ng/ml; accuracy: −1.87%; precision: ±2.75%). Fig. 1 shows chromatograms of drug-free plasma, plasma spiked with defined amount of norfloxacin and volunteer plasma after oral intake of norfloxacin.

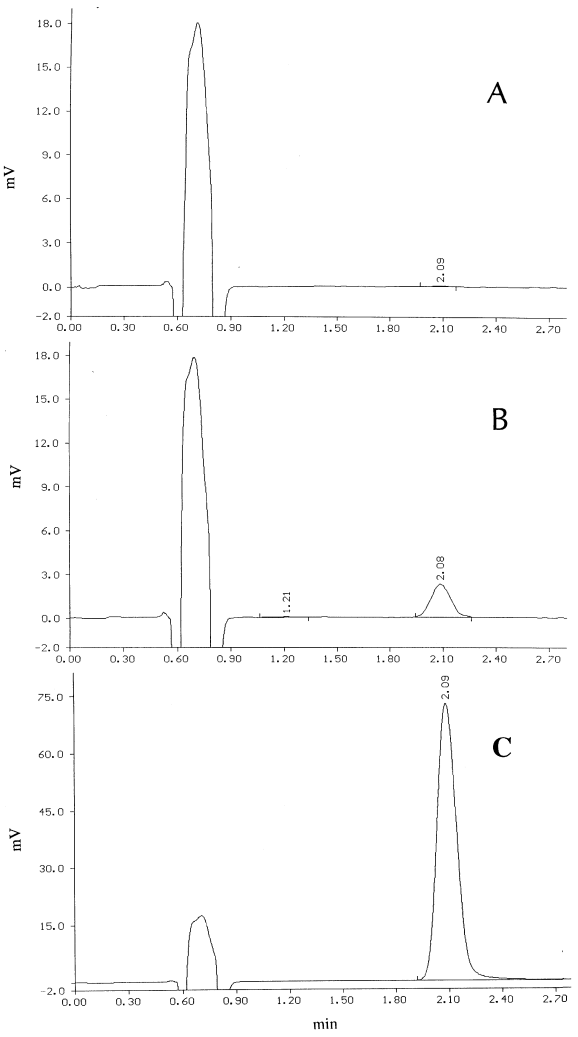

Fig. 1. Chromatograms of (A) blank human plasma, (B) a plasma sample taken from a volunteer 8 h after oral intake of 400 mg norfloxacin (concentration 91 ng/ml) and (C) a calibration sample spiked in plasma at a concentration of 2460 ng/ml.

3.2. Assay specificity

The specificity of the method was determined by screening blank plasma from six different volunteers. Also the following drugs do not interfere in this assay: metronidazol, doxycycline, ampicillin, lansoprazole, penicillin V, minocycline, clavulanic acid, omeprazole, erythromycin, amoxicillin, trimethoprim, and acyclovir.

3.3. Assay precision and accuracy

The intra-day precision and accuracy was determined by analysing four aliquots of three quality-control samples within one run. Means, relative standard deviation (±%) and accuracy were determined. The intra-day precision was good and consistent with values of ±4.14% for a concentration of 70.3 ng/ml and ±1.90% and ±0.37% (each $n=4$) for a concentration of 316.4 and 2109.2 ng/ml (see Table 1).

3.4. Extraction recovery from plasma

Recovery was measured by comparison of back-calculated concentrations (using regression parameters of calibration graph) of nonextracted solutions containing the theoretical amount versus extracted standards of spiked plasma. The recovery of norfloxacin was determined as 88.3% at 29.1 ng/ml, 88.0% at 273.0 ng/ml and 86.5% at 2461.5 ng/ml.

3.5. Peak shape

Different types of reversed-phase materials and mobile phase mixtures were tested during method development. In particular, the mobile phase showed a great influence on the peak shape. Different ion-pair reagents were tested e.g. tetramethylammonium-fluoride. Furthermore, methanesulfonic acid, citric acid and perchloric acid were tested. Using Nucleosil $3C_{18}$ as stationary phase and only methanol and the high molarity of perchloric acid–triethylamine as mobile phase resulted in a satisfying peak shape. The high molarity of the acid resulted in a rather low pH value, but during analysis of about 800 plasma and urine samples, no change in the retention time and peak shape was observed.

Table 1
Intra-day precision of quality control samples in plasma concentration spiked

Concentration spiked (μg/ml)	R.S.D. (%)	Accuracy (%)
70.3	±4.14	−6.38
316.4	±1.90	−9.47
2109.2	±0.37	−5.18

3.6. Urine

The HPLC method for measurement of norfloxacin in human plasma was tested for the suitability to quantitate norfloxacin in human urine. The only change of the assay conditions refers to the sample work-up: instead of deproteinizing using acetonitrile, the urine samples were diluted with water. As the validation in plasma samples has proven the good performance of the method in general, the validation procedure for measurement of urine samples was limited to the fundamental assay parameters.

The calibration graph showed a good linearity with a coefficient of correlation (R^2) of greater than 0.999 in the calibration range of 5.1–410 μg/ml of urine. The relative standard deviations (R.S.D.%) ranged between ±0.08 and ±1.05%. The accuracy for the mean values ranged between −1.61 and 2.96%. Fig. 2 shows chromatograms of drug-free urine and human urine spiked with two defined amounts of Norfloxacin.

3.7. Stability experiments

Evaluation of the stability of spiked plasma samples was done by comparing the mean value at the respective condition with an initial value (results see Table 2). For the determination of the counter stability (stability during sample preparation) calibration samples were thawed at defined timepoints (22 h, 6 h and 1 h before sample clean-up, treatment D). Stability of norfloxacin in native plasma was given over 22 h. Two freeze–thaw cycles did not alter the concentration of norfloxacin in human plasma (treatment A). Stability was also tested after sample preparation without and with mixing the acetonitrile supernatant with perchloric acid and triethylamine (0.1 and 0.02 *M* in water, respectively). No instability of norfloxacin was observed over a time period of 2 h after precipitation with acetonitrile (treatment B) and 21 h at room temperature after precipitation with acetonitrile and mixing with perchloric acid and triethylamine (0.1 and 0.02 *M* in water, respectively, stored in darkness, treatment C).

Stability in urine was tested in the concentration range of 7.5–600 μg/ml at two freeze–thaw cycles (accuracy −0.9 to 1.2%) and at room temperature over 4 h (accuracy −0.7 to +4.1%) and 20 h

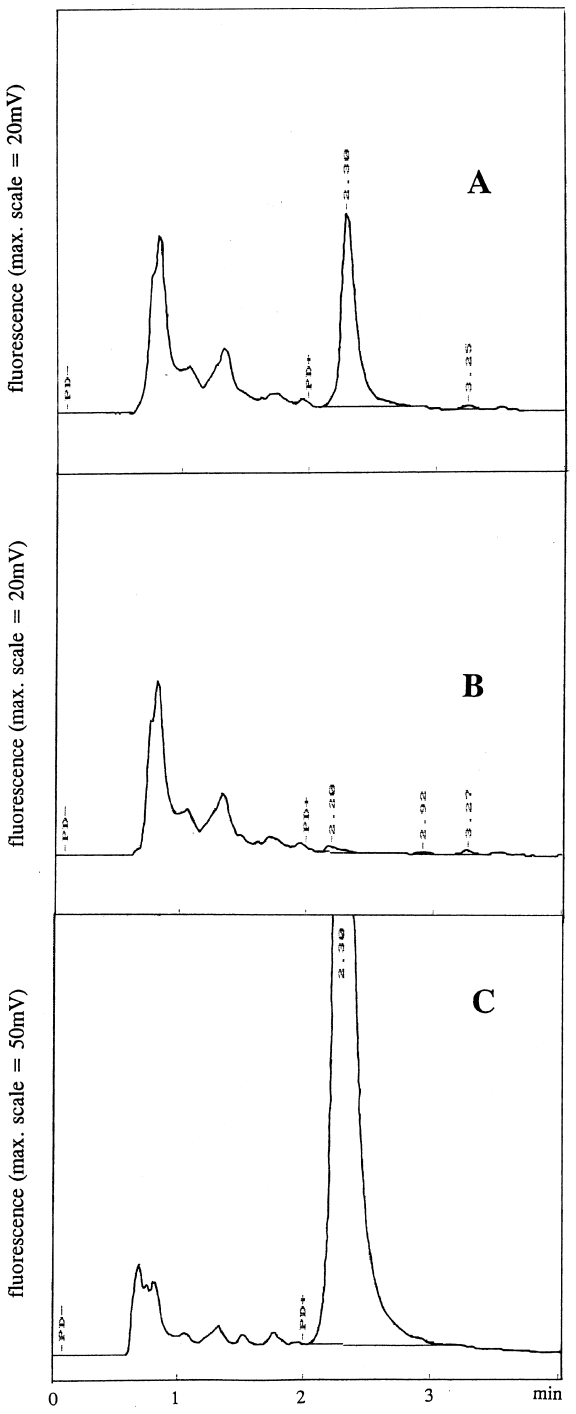

Fig. 2. Chromatograms of (A) a calibration sample spiked in urine at a concentration of 15.2 μg/ml, (B) blank human urine and (C) a calibration sample spiked in plasma at a concentration of 410 μg/ml.

Table 2
Stability of norfloxacin in plasma under different treatments

Concentration (ng/ml)	Deviation from the initial value (rel.%) Treatment			
	A	B	C	D
30.4	−4.97	−5.24	lost	−3.50
91.1	3.52	2.11	−1.37	−0.51
273.3	3.85	5.17	−1.84	−1.65
820.0	2.82	7.09	−1.59	+1.88
2460.0	1.55	6.08	−1.34	+0.85

A: Two thaw-and-freeze cycles; B: 2 h at room temperature after precipitation with acetonitrile; C: 21 h at room temperature after precipitation with acetonitrile and mixing with perchloric acid and triethylamine (0.1 and 0.02 *M* in water, respectively) in darkness; D: 22 h at room temperature in plasma.

(accuracy −3.8 to +0.4%). No substantial instability was observed under the described conditions.

4. Conclusions

This paper describes a sensitive, selective and reliable HPLC assay for norfloxacin in human plasma and urine. The method includes protein precipitation with perchloric acid for plasma samples and simple dilution for urine samples. Although a high molarity of perchloric acid was used in the mobile phase (and, hence, low pH values) no decrease of the chromatographic performance was observed over the approx. 2-week period of analyses. The method has been used successfully for the measurement of samples derived from human pharmacokinetic studies with single dose administration of norfloxacin. More than 100 samples could be treated daily by manual sample preparation and an automated HPLC system. Fig. 3 shows a semilogarithmic plot of the plasma concentrations in a volunteer after administration of a single oral dose of a test and a reference formulation of 400 mg Norfloxacin.

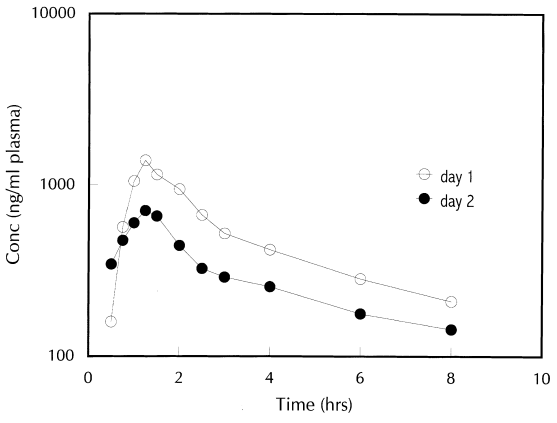

Fig. 3. Pharmacokinetic profile of one volunteer after oral administration of two different galenic formulations of norfloxacin (each 400 mg).

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