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Short communication

Determination of ofloxacin in human plasma using high-performance liquid chromatography and fluorescence detection

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Abstract

A new method for the determination of ofloxacin in human plasma was developed. Plasma proteins were precipitated with acetonitrile, the supernatant concentrated and injected into a reversed-phase C_{18} column. Enoxacin was used as an internal standard. The fluorimetric detection was performed at 282 nm for excitation and 450 nm for emission. Limit of quantitation was 20 ng/ml and the calibration curve was linear up to 6900 ng/ml.

1. Introduction

Ofloxacin is a fluoroquinolone derivative which shows a broad antimicrobial spectrum. Several HPLC methods for ofloxacin determination in biological fluids were described in the literature [1–7]. Most of them use fluorescence detection in order to achieve high sensitivity and selectivity and a reversed-phase column. The methods differ mainly in the selection of the internal standard and in sample preparation technique. Time-consuming liquid–liquid extraction [1–4,7] and more expensive solid-phase extraction [5] or column switching [6] can be found in the literature but direct protein precipitation was not employed in spite of its simplicity. Moreover, many methods use structurally unrelated compounds or commercially unavailable derivatives of ofloxacin [3,5] as the internal standard.

We have developed a rapid and simple method for ofloxacin determination in plasma which can be applied to pharmacokinetic studies in humans after a single oral dose. The sample preparation technique is protein precipitation and enoxacin is used as an internal standard.

2. Experimental

2.1. Chemicals

Ofloxacin was a gift from Léčiva, Prague, Czech Republic. Enoxacin (internal standard) was purchased from Sigma-Aldrich (Prague, Czech Republic), acetonitrile and tetrahydrofuran (both for HPLC) were obtained from Riedel-de-Haen (Seelze, Germany). Triethylamine (TEA) and dihydrogenpotassium phosphate were of the analytical grade and were purchased from Merck (Darmstadt, Germany).

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o-Phosphoric acid was supplied by Lachema (Brno, Czech Republic).

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of a membrane degasser, pump ConstaMetric 4100, automatic sample injector AutoMetric 4100, fluorimetric detector FL2000 and a data station with LCTALK software, version 2.02.

The separation was performed on a Separon SGX C_{18} 7 μm, 150 × 3 mm I.D. glass column (Tessek, Prague, Czech Republic). The mobile phase consisted of 5.5% tetrahydrofuran and 94.5% of (0.06 *M* dihydrogenpotassium phosphate, pH 2.6 adjusted with o-phosphoric acid–TEA, 97:3, v/v). Sodium azide (50 mg/l) was added to the buffer to prevent bacterial growth. The flow-rate was 0.7 ml/min at 25°C. The fluorimetric detector was operated at the following wavelengths: excitation 282 nm, emission 450 nm. The time constant was set to 2 s.

2.3. Preparation of standard solutions

Six mg of ofloxacin was dissolved in 25 ml of methanol and solutions for preparation of calibration curve and quality control samples were obtained by serial dilutions with methanol. These solutions were added to drug-free human plasma containing heparin as an anticoagulant in volumes not exceeding 4% of the plasma volume. Enoxacin (4.5 mg) was dissolved in 25 ml of methanol. All solutions were stored at 4°C and protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −20°C. The thawing was allowed overnight at 4°C. Fifty μl of internal standard solution (9 μg) was added to 1 ml of plasma, the tube was briefly shaken and 3 ml of acetonitrile were added. The tube was shaken for 30 s at 1000 rpm and centrifuged 10 min at 2600 *g*. Three ml of the sample were transferred to another tube and evaporated to dryness under nitrogen at 60°C. The sample was reconstituted in 100 μl of the phosphate buffer (see mobile phase, above) before analysis, transferred to a 100-μl polypropylene autosampler vial and 30 μl was injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed daily in the range 20.14–6896 ng/ml to encompass the expected concentrations in measured samples. It was obtained by weighted linear regression (weighing factor $1/y^2$): the ratio of ofloxacin peak area to enoxacin peak area was plotted vs. ofloxacin concentration.

3. Results and discussion

3.1. Sample preparation

Initially we tried liquid–liquid extraction with dichloromethane [1,2] but it led to results with an unsatisfactory intra-assay reproducibility (R.S.D. 10% at 710 ng/ml, $n = 7$). The variability can be attributed to the amphoteric character of both fluoroquinolones. These compounds should be extracted at their isoelectric points (p*I*) and any deviation from the p*I* value causes a change in the extraction recovery. Moreover, the p*I* values of the studied compounds are similar but not identical (6.97 and 7.38 for ofloxacin and enoxacin, respectively [8]) and this difference is an additional source of variability. We therefore used the procedure with the protein precipitation with acetonitrile (no pH adjustment is required) followed by the evaporation of the supernatant which produced satisfactory results in a short time.

3.2. Chromatography

The blank sample chromatogram (Fig. 1) showed only one major peak at 4 min which was completely separated from the internal standard. The chromatogram of a plasma sample after administration of ofloxacin to a volunteer is

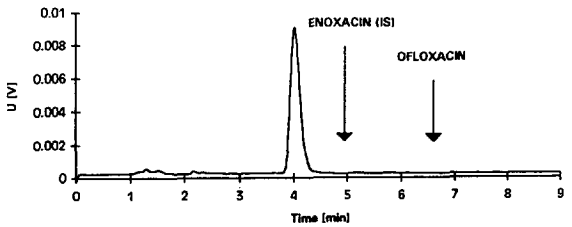

Fig. 1. Typical chromatogram of drug-free human plasma. The arrows indicate the retention times of ofloxacin and enoxacin (internal standard).

shown in Fig. 2. The retention time of enoxacin and ofloxacin was 5.0 and 6.7 min, respectively. The column efficiency expressed by the number of theoretical plates was 2000 and 1600 for enoxacin and ofloxacin, respectively. Tailing of the peaks which is reported to be a serious problem for ofloxacin was successfully suppressed by the addition of TEA to the mobile phase. The TEA concentration was higher than usually used for this purpose but we did not encounter any problems with retention time stability or column life. More than 400 samples were successfully analysed on the same column.

3.3. Limit of detection and quantitation

The limit of detection (signal-to-noise ratio 3:1) for ofloxacin was 2 ng (i.e., 8 ng/ml). This value was sufficient for our purposes, but it can be easily lowered by injecting a larger volume of the sample or by adjusting detection wavelengths of the fluorescence detector to obtain maximum response of ofloxacin (excitation wavelength 296 nm and emission wavelength 500 nm). However, under such conditions the response of the fluorimetric detector became non-linear at higher concentrations of ofloxacin (above 1 μg per injection) and also the response of enoxacin was very week.

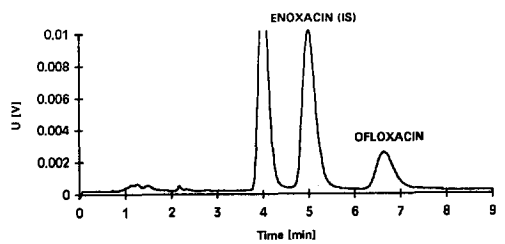

Fig. 2. Chromatogram of a plasma sample from a volunteer 12 h after administration of 200 mg of ofloxacin. The measured concentration of ofloxacin was 331 ng/ml.

The limit of quantitation (defined as concentration at which precision and accuracy are better than 20%) was 20 ng/ml.

3.4. Linearity

In the pharmacokinetic applications the range of linearity has to be sufficiently large. In the previous methods the calibration range was split into two ranges which required the measurement of more calibration samples [2,3] or the linearity range was too narrow [1,4–7].

We prefer to use a single calibration curve instead of tedious dilution and re-analysis of samples with a high concentration of ofloxacin. By proper selection of sample preparation technique and detection wavelengths (see above) we obtained a single linear calibration curve in the range 20–6900 ng/ml using weighted linear regression; the typical value of the correlation coefficient was higher than 0.9999.

3.5. Precision and accuracy

Intra-day precision of the method is illustrated in Table 1. Precision, characterised by the relative standard deviation, was 8% at 20 ng/ml and better than 3% at higher concentrations.

Inter-day precision and accuracy was evaluated using analysis of quality control samples which were prepared in advance, stored at −20°C and

Table 1
Intra-day precision

Concentration (ng/ml)	n	R.S.D. (%)
20.14	7	7.5
39.93	7	2.6
150.3	7	1.7
401.9	7	2.1
1589	7	2.7
3934	7	1.5
7742	5	1.2

Table 2
Inter-day precision and accuracy

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Bias (%)	R.S.D. (%)
69.80	8	67.65	−3.1	10.5
312.6	8	299.9	−4.1	3.7
1194	9	1231	3.1	3.1
4994	8	4977	−0.3	4.2

n = Number of days.

daily measured with each set of unknown samples. The respective data are given in Table 2, the precision was better than 11% and the inaccuracy did not exceed 5%.

3.6. Sample stability

The processed samples in the phosphate buffer were stable at least 20 h at room temperature. The plasma samples were stable at least 3 weeks at −20°C with two thaw and freeze cycles (data not shown, see also paper by Fabre et al. [7]).

4. Conclusions

A method has been developed for the rapid and precise determination of ofloxacin in human plasma. The method was useful in the acquisition of pharmacokinetic data up to 36 h after oral administration of ofloxacin (200 mg).

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