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Development and validation of high-perfomance liquid chromatographic method for determination of ofloxacin and lomefloxacin in human plasma

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Abstract: A high-performance liquid chromatographic method for the determination of ofloxacin and lomefloxacin in human plasma has been developed and validated. The effect of organic modifiers on the retention of the investigated drugs was investigated. A simple isocratic chromatographic assay with UV-detection at 280 nm was performed on a Hibar Lichrospher 100 RP 8 column (250 × 4.6 mm, 5 μm. Merck, Germany) using a mixture of acetonitrile and 0.5 % triethylamine in water (pH adjusted to 2.5 with H_3PO_4) (15:85, V/V) as the mobile phase at flow rate of 1.2 mL min^{-1}. The calibration curves were linear in the concentration ragne of 0.5 – 6.0 μg mL^{-1} for ofloxacin and 0.2-4.5 μg mL^{-1} for lomefloxacin.

Keywrods: ofloxacin, lomefloxacin, high-performnce liquid chromatography.

INTRODUCTION

The recent introduction of fluorinated 4-quinolones, such as ofloxacin [(±) -9-fluoro-2,3-dihydro-methyl-10-(4-methyl-1-pierazinyl)-7-oxo-7oxo-7H-pyrido (1,2,3 -de)-1,4-benzoxazine-6-carboxylic acid, $C_{18}H_{20}FN_3O_4$], represents a particularly important therapeutic advance, since these agents have broad antimicrobial activity and are effective after oral administration for the treatment of a wide variety of infections diseases.[1] Lomefloxacin [(±)-1-ethyl-6,8-difluoro-7-(3-methyl-1-piperazinyl)-4ox-3-quinolinecarboxylic acid, $C_{17}H_{19}F_2N_3O_3$] is a fluoroquinolone antibacterial with actions and uses similar to those of ciprofloxacin. It is given orally as the hydrochloride for the treatment of susceptible infections, including bronchitis due to Haemophilus influenzae or Moraxella catarrhalis and urinary tract infections.

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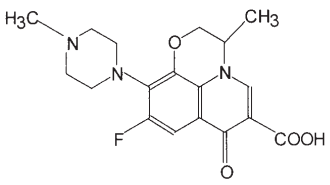
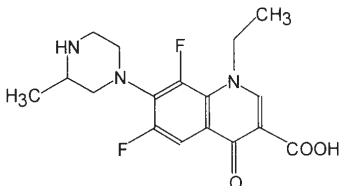


Ofloxacin (M_W = 361.4)



Lomefloxacin (M_W = 351.3)

The determinations of ofloxacin and lomefloxacin in pharmaceutical preparations by high performance liquid chromatography have been the subject of several investigation (HPLC).[2,3] A number of assay methods for ofloxacin and lomefloxacin in biological fluids have been reported.[4–22] Many authors determined ofloxacin or lomefloxacin in blood plasma or serum after protein precipitation using perchloric acid,[4,5] acetonitrile[6,7,22] or methanol.[8] These methods gave poor separation of the investigated drugs from the endogeneous interferences of the blood plasma and serum and the recorveries were realtively low. In the published methods, liquid–liquid extraction with dichloromethane,[9,10] chloroform,[11,19] a mixture of chloroform–2-propanol–diethyl ether[12] or ethyl acetate–2-propanol[21] as solvents were used for sample preparation. The disadvantage of these methods employing liquid–liquid extraction of ofloxacin and lomefloxacin from biological fluids is that they involve several steps and long times are required.

Other investigators performed the separation of ofloxacin or/and lomefloxacin from human plasma or serum using the HPLC column switching method.[13,15] The separation of ofloxacin and lomefloxacin from serum or plasma with solid-phase extraction has been reported by several investigators.[16–18] These methods are expensive, take time, a large consumption of reagent and an HPLC system with two pumps. Krol *et al.*[23] reported a HPLC method with fluorescent detection for the determination of ciprofloxacin and ciprofloxacin metaboliltes in body fluids.

The aim of this study was to develop a method which enabled the determination of ofloxacin and lomefloxacin (racemic mixtures, not individual enantiomers) in human plasma using HPLC with UV detection without the need for the development of separate and distinct methods for each agent. For the purpose of minimizing the variability caused by sample pretreatment, the method of internal standardization for the quantification of these drugs is suggested. In order to fulfil the aim, the method was first developed for the separation and determination of the quinolones concentrations by optimizing the experimental parameters and determining the linearity for the two fluoroquinolones. Then, the method for the determination of the ofloxacin and lomefloxacin concentrations was validated by evaluating the recovery, selectivity, linearity, precision and accuracy. There was no need to develop a method which could provide for the selective determination of ofloxacin and its microbiologically active metabolite (desmethyl ofloxacin) because ofloxacin is not extensively metabolized and only low concentrations of the active ofloxacin metabolite are attained in serum. Finally, the method was emplo-

yed for the determination of ofloxacin in plasma samples obtained from healthy volunteers.

EXPERIMENTAL

Materials

Ofloxacin working standard was supplied by Selectchemie AG, (Switzerland) and lomefloxacin was purchased from Sigma (Germany). The internal standard of ciprofloxacin was kindly supplied by Morepen Laboratories Limited (India). HPLC Grade acetonitrile and methanol were purchased from Across Organics (Belgium). Triethylamine (TEA), *o*-phosphoric acid, potassium dihydrogen phosphate and trichloroacetic acid were obtained from Merck (Germany).

Instrumentation

HPLC was performed using a Perkin–Elmer liquid chromatography system (USA) consisting of a pumpe PE LC series 200, an autosampler PE LC ISS Series 200, a diode array detector PE LC 235 C and a column oven PE model 101. The chromatographic system was controlled by the software package Turbochrom Version 4.1 plus and the UV-spectrometric data were produced by the program TurboScan Version 2.0.

In order to develop a convenient and easy-to-use method for the simultaneous determination of ofloxacin and lomefloxacin in human plasma, and with respect to the location and shape of the peaks of investigated quinolones in the corresponding chromatograms, different column packings, including C8, C18 and RP-select B, with different lengths and particle sizes were tested. The final choice of the stationary phase giving satisfying resolution and run time was a Hibar Lichrospher 100 RP 8, 250 × 4 mm I.D. (5 μm, particlel size), protected by a guard column Lichrospher 100 RP 8, 4 x 4 mm (5 μm). A flow-rate of 1.2 mL min^{-1} was used for the separations of ofloxacin, lomefloxacin and internal standard with UV detection at 280 nm. A column temperature of 25 ^{o}C was used with an injection volume of 120 μL.

Preparation of standards and plasma samples

Stock solutions of ofloxacin, lomefloxacin and the internal standard (ciprofloxacin) were prepared at a concentration of 100 μg mL^{-1}. The stock solution of ofloxacin was prepared by dissolving the appropriate amount in 1 mL of 0.5 mol L^{-1} HCl and 49 mL of water. The stock solution of lomefloxacin was prepared by dissolving the appropriate amount in 1 mL of 0.1 mol L^{-1} NaOH and 49 mL of water and the working stock solution of ciprofloxacin was prepared in water. These solutions were prepared monthly and stored at 4 ^{o}C. No change in stability over a period of 1 month was observed. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

Human plasma was prepared from heparinized whole blood samples. The blood samples were collected from healthy volunteers, who later participated in a bioequivalence study, and stored at –20 ^{o}C. After thawing, the samples were spiked daily with working solutions of ofloxacin, lomefloxcin and internal standard.

Plasma samples were precipitated using a mixture of phosphoric acid, trichloroacetic acid and acetonitrile, similarly to a previously reported procedure.[23] Namely, a 0.5 mL aliquot of plasma sample was diluted with 0.1 mL of 0.1 mol L^{-1} phosphoric acid and 0.3 mL of 5.0 mol L^{-1} trichloroacetic acid–acetonitrile (1:1, V/V) solution. The mixture was vortexed and diluted again with 0.1 mL of acetonitrile and 0.3 mL of water. The final solution was vortexed and centrifuged for 10 min at 10000 g. An aliquot of the supernatant was transferred into a glass autosampler vial for HPLC analysis.

Calibration curves

Typical calibration curves were constructed with six blank plasma samples spiked with appropriate amounts of the standard solutions. The calibration range was 0.5–6.0 μg ofloxacin and 0.2–4.5 μg lomefloxacin per mL of plasma. The standard samples were prepared according to the

procedure used for the unknown samples. The calibration curves were obtained by plotting the ratios of the peak heights of ofloxacin and lomefloxacin to the internal standard *versus* their concentration in μg/mL. The regression equations were calculated by the least-squares method.

Quality control (QC) samples were prepared at low, medium and high levels in the same way as the plasma samples for calibration.

RESULTS AND DISCUSSION

Several variables of the HPLC method with respect to their effect on the separation of ofloxacin, lomefloxacin and the internal standard from the matrix were investigated. In extensive preliminary experiments, a series of aqueous mobile phase with different pH values in combination with different organic modifiers were tested.

Method development

The amount of organic modifier present in the mobile phase influences analytes which are retained predominantly by adsorption onto the stationary phase. The results obtained by varying the acetonitrile concentration in the mobile phase (0.5 % (V/V) triethyl amine, TEA, pH 2.5 adjusted with H_3PO_4 over the range 10–20 % are shown in Fig. 1. These data were used to determine the optimal amount of organic modified which should be used for the separation of ofloxacin, lomefloxacin and the internal standard, ciprofloxacin. The best results (good separation of the three peaks, short analysis time) can be obtained when the percentage of acetonitrile in the mobile phase is 15 %.

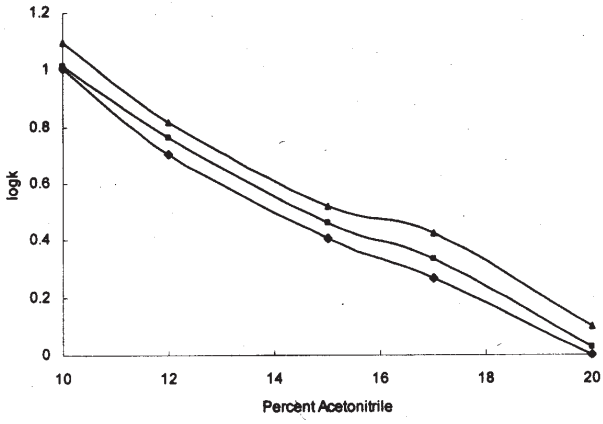


Fig. 1. The effect of the acetonitrile concentration on the retention of the analytes (◆ – ofloxacin; ▲ – lomefloxacin; ■ – internal standard).

An additional study was also performed to determine the effect that the percentage of TEA in the mobile phase had on the retention of the analytes. The results that were obtained over a TEA percentage range of 0.0 % to 1.0 % in the mobile phase (TEA, pH 2.5 adjusted with H_3PO_4 and 15 % acetonitrile) are shown in Fig. 2. The retention times of ofloxacin, lomefloxacin and internal standard decreased with increasing percentage of TEA in the mobile phase. Also, before the addition

of TEA, the investigated drugs showed peak tailing. The peak tailing may be attributed to hydrogen bonding between the free silanols of the stationary phase and the amino groups on the quinolones. In fact, TEA acts like an organic modifier. On the other hand, with mobile phase containing TEA, peaks of much better shape were obtained. These improved peak shapes may be due to TEA blocking the fixed silanol sites. Also, adsorbed TEA would prevent hydrogen bonding with the amino groups on the investigated quinolones.

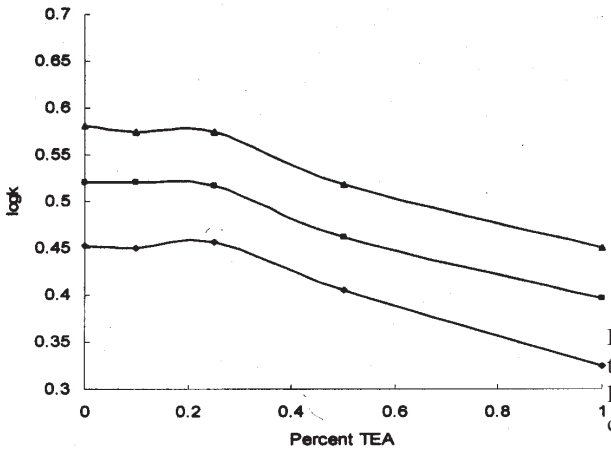


Fig. 2. The effect of the TEA concentration on the retention of the analytes (◆– ofloxacin; ▲– lomefloxacin; ■– internal standard).

From these data, it was demonstrated that a mobile phase consisting of 0.5 % TEA, pH 2.5 adjusted with H_3PO_4 and 15 % acetonitrile would provide good retention for ofloxacin, lomefloxacin and the internal standard, as well as an acceptable runtime of less than 10 min for the separation.

A typical chromatogram of the standard solutions of ofloxacin, lomefloxacin and internal standard produced by the developed HPLC method is shown in Fig. 3 a. The retention times of ofloxacin, internal standard (ciprofloxacin) and lomefloxacin are 6.7, 7.5 and 8.25 min, respectively.

In Fig. 3a, a characteristic base line disturbance can also be seen at about 1.9 min following injection. This rapid upwards or downwards deflection of the trace from is caused by the difference in the compositions of the sample solution and the mobile phase. It is safe to assume that this corresopongs to t_0.

In addition, different reagents (acetronitrile, perchloric acid, trichloroacetic acid) were used for protein precipitation in order to obtain satisfactory values for recovery of oflowacin, lomefloxacin and internal standard. The plasma samples were treated with an equal volume of acetonitrile (procedure 1) or 6 % trichloroacetic acid (procedure 2). For the precipitation of the plasma proteins to 0.25 mL of spiked plasma sample, 0.05 mL of 20 % perchloric acid were added (procedure 3) or a mixture of phosphoric acid, trichloroacetic acid and acetonitrile (procedure 4). The extraction recoveries were calculated by comparing the heights of the ofloxacin, lomefloxacin and internal standard peaks for two concentration levels

(n - β for each level for ofloxacin and lomefloxacin, $n = 6$ for the internal standard) with those resulting from the direct injection ($n = 3$, working solutions) of the theoretical amount of drugs (= 100 % recovery). The results of this investigation are presented in Table 1. As can be seen, satisfactory values for the recovery of ofloxacin, lomefloxacin and the internal standard were obtained when the plasma samples were prepared according to procedure 4 (Table I).

TABLE I. Absolute recoveries of ofloxacin, lomefloxacin and the internal standard from spiked plasma samples

	Mean recovery/%			
	Procedure 1	Procedure 2	Procedure 3	Procedure 4
γ^* (Ofloxacin)/μg mL^{-1}				
1.0	12.05	68.31	71.97	100.72
2.0	14.14	74.20	73.63	93.87
γ(Lomefloxacin)/μg mL^{-1}				
1.0	13.25	77.16	79.43	102.77
2.i	13.03	79.82	84.26	105.18
γ(Internal standard)/μg mL^{-1}				
2.0	11.69	67.39	67.44	95.26

γ^* –Mass concentration

Under the described chromatographic conditions, the ofloxacin, lomefloxacin and internal standard peaks were well resolved. Endogenous plasma components did not intefere with any of the peaks. Typical chromatograms of blank plasma in comparison to spiked samples are shown in Fig. 3b, c.

The developed HPLC method was used for the analysis of plasma samples from healthy volunteers after oral administration of ofloxacin. A typical chromatogram of the plasma sample of a patient after administration of 400 mg of ofloxacin is shown in Fig. 4.

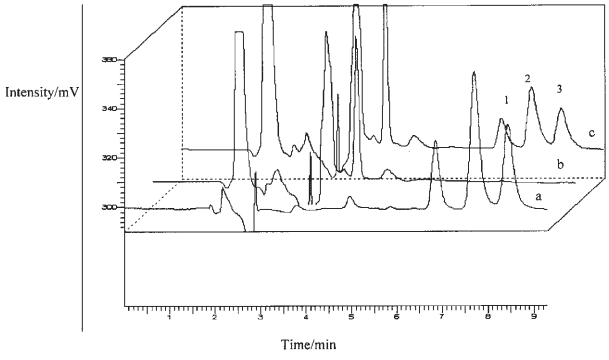


Fig. 3. Chromatograms of standard solutions (a) of ofloxacin, lomefloxacin and internal standard (2 μg mL^{-1}); 1–ofloxacin, 2–internal standard, 3–lomefloxacin, blank (b) and spiked plasma (c) samples containing 2 μg mL^{-1} of ofloxacin and internal standard and 1.5 μg mL^{-1} of lomefloxacin.

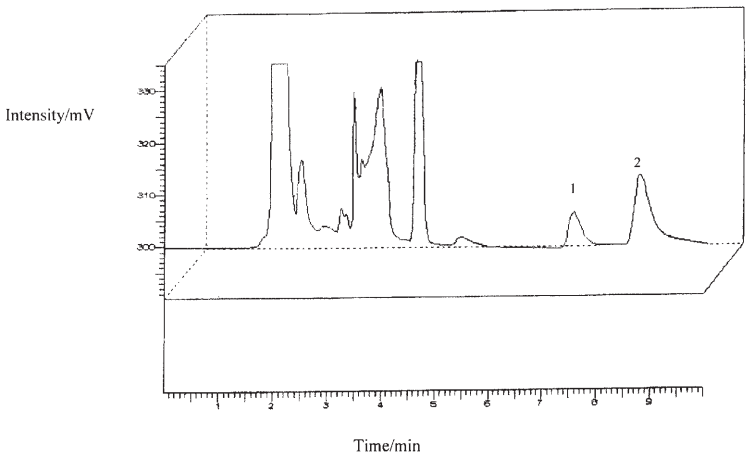


Fig. 4. Chromatogram of a plasma sample from a healthy volunteer 40 min after administration of ofloxacin; 1–ofloxacin, 2–internal standard.

Method validation

Linearity. The linearity was tested on three different days at five concentration points ranging from 0.5 to 6.0 μg mL^{-1} of ofloxacin and 0.2–4.5 μg mL^{-1} of lomefloxacin in plasma samples. The plasma samples were spiked with the internal standard (cirpofloxacin) at a concentration of 2.0 μg mL^{-1}. The respective regression equations including the values for the standard errors of the intercept and slope were: $y = (5074 \pm 1.79)y + 102.93 \pm 1.29$ for ofloxacin and $y = (9935.4 \pm 94.23)y - 1111 \pm 140.86$ for lomefloxacin. The correlation coefficients were 0.9999 and 0.9951, respectively.

TABLE II. Intra- and inter- day precision data

Nominal concentration/μg mL^{-1}	Intra-day		Inter-day	
	Mean (n=3) observed concentration/μg mL^{-1}	Relative standard deviation/%	Mean (n = 9) observed concentration/μg mL^{-1}	Relative standard deviation/%
Ofloxacin				
0.5	0.51	1.13	0.52	2.43
1.0	1.02	1.96	1.05	2.0
2.0	2.01	4.76	1.98	4.55
4.0	4.02	0.5	4.07	0.64
6.0	6.05	0.33	6.09	0.99
Lomefloxacin				
0.2	0.23	4.35	0.24	4.13
0.8	0.82	2.44	0.78	3.3
1.5	1.42	1.07	1.47	2.2
3	3.05	0.33	3.02	1.24
4.5	4.54	0.56	4.49	0.65

Precision. On one day and on 3 different days, spiked samples of each concentration used for the construction of the calibration curves were prepared in triplicate and analyzed by the proposed HPLC method. Then, the corresponding coefficients of variation were calculated. The intra- and inter-day variations of the method throughout the linear range of concentrations are shown in Table II. These data indicate the considerable degree of precision and reproducibility of the method, both during one analytical run and between different runs.

Accuracy. Intra- and inter-day accuracies were determined by measuring the plasma quality of control samples at low, middle and high concentration levels of ofloxacin and lomefloxacin. An indication of the accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration. The accuracy and recovery data are presented in Table III. The relative errors at all three studied concentrations for ofloxacin and lomefloxacin were less than 4.8 % and it is obvious that the method is remarkably accurate, which ensures that reliable results are obtained.

TABLE III. Intra- and inter- day accuracy and recovery data

	Intra-day			Inter-day		
Nominal concentration μg mL^{-1}	Mean (n = 3) observed concentration μg mL^{-1}	Recovery %	Relative error/%	Mean (n = 9) observed concentration μg mL^{-1}	Recovery %	Relative error/%
Ofloxacin						
0.8	0.82	102.5	2.5	0.83	103.8	3.75
1.2	1.17	97.5	– 2.5	1.19	99.2	– 0.83
2.5	2.59	103.6	3.6	2.62	104.8	4.8
Lomefloxacin						
0.5	0.52	104.0	4.0	0.51	102.0	2.0
1.0	1.02	102.0	2.0	0.98	98.0	–2.0
2.0	2.05	102.5	2.5	2.02	101.0	1.0

Limit of quantifiacion. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15 % (n = 5) and an accuracy of ±15 % (n = 5). The limits of quantification were found to be 0.5 μg mL^{-1} for ofloxacin and 0.2 μg mL^{-1} for lomefloxacin.

Stability of ofloxacin and lomefloxacin in plasma samples . The stabilites of ofloxacin and lomefloxacin in plasma were investigated using spiked samples at two different concentration levels, prepared in duplicate. Spiked samples were analysed after different storage conditions: immediately, after standing in the autosampler for 2, 12 and 24 h, after one and two freeze/thaw cycles and after two weeks stored at –20 °C. The results from this investigation show that ofloxacin and lomefloxacin added to plasma were stable under different storage conditions.

Ruggedness. The ruggedness was tested on a second HPLC column of the same type by determining the linearity, precision and accuracy. The linearity test was performed at five concentration points for ofloxacin and lomefloxacin in plasma in the concentration range from 0.5 to 6.0 μg mL^{-1} and 0.2 to 4.5 μg mL^{-1}, respectively. The regression equations were: for ofloxacin $y = 5802.4\,y + 35.124$ and for lomefloxacin $y = 9663.6\,y - 1020.3$. The correlation coefficients were 0.9993 and 0.995, respectively. The intra-day precision and accuracy were determined by measuring three series of plasma quality control samples. The relative standard deviations at all three concentrations studied for ofloxacin and lomefloxacin were less than 3.4 %. The relative errors ranged from 0.77 to 6.66 % of the nominal concentrations of the investigated drugs. As can be seen, the results of this assessment are very similar to those obtained from the previous investigation of the first HPLC column. This means that this HPLC method of the simultaneous determination of ofloxacin and lomefloxacin in spiked human plasma samples is rugged.

CONCLUSION

The developed HPLC method employing protein precipitation for the sample preparation is simple and convenient for the simultaneous determination of ofloxacin and lomefloxacin in plasma samples using the internal standard method. The typical assay time is about 10 min. The proposed method is simple, rapid and provides an efficient clean up of the complex biological matrix and high recovery of ofloxacin and lomefloxacin. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Also, the method can be used to monitor ofloxacin and lomefloxacin levels in clinical samples.

ИЗВОД

РАЗВОЈ И ВАЛИДАЦИЈА МЕТОДЕ ЗА ОДРЕЂИВАЊЕ ОФЛОКСАЦИЈА И ЛОМЕФЛОКАСЦИНА У ХУМАНОЈ ПЛАЗМИ ПОМОЋУ ВИСОКОЕФИКАСНЕ ТЕЧНЕ ХРОМАТОГРАФИЈЕ

ДРАГИЦА ЗЕНДЕЛОВСКА и ТРАЈЧЕ СТАФИЛОВ

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Развијена и валидирана је метода за одређивање офлоксацина и ломефлоксацина у хуманој плазми помоћу високоефикасне течне хроматографије. Испитиван је утицај органских модификатора на ретенцију испитиваних лекова. Примењено је изократно хроматографско одвајање са UV детекцијом на 280 nm на Hibar Lichrospher 100 RP 8 хроматографској колони (250 x 4,6 mm, 5 μm, Merck, Немачка) користећи смешу од ацетонитрила и 0,5 % триетил амина (pH do 2,5) (15:85, V/V) (15:85, V/V*)* као мобилну фазу са протоком од 1,2 mL min^{-1}. Калибрациони дијаграми су линеарни у концентрационом подручју од 0,5–6,0 μg mL^{-1} за офлоксацин и 0,2–4,5 μg mL^{-1} за ломефлоксацин.

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