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THIN-LAYER CHROMATOGRAPHIC SEPARATION AND IN SITU FLUORIMETRIC DETERMINATION OF OFLOXACIN IN PLASMA AND PLEURAL FLUID[a]

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SUMMARY

A thin-layer chromatographic assay for the determination of ofloxacin in human plasma and pleural fluid is described. After extraction of ofloxacin from samples with dichloromethane, chromatography was performed on thin-layer plates (silica gel) with a mobile phase consisting of ethanol and water; the tank atmosphere was equilibrated with concentrated ammonia. The precision of the assay could be considerably increased along with the measured fluorescence intensity of ofloxacin by spraying the plate with a citric acid solution and dipping it into paraffin or using a mixture of both components. Peaks were quantified by densitometric evaluation of the chromatograms. The method shows a very low limit of detection (1 ng/ml) as well as good precision and linearity in the range 0.001–2.0 μg/ml for both plasma and pleural fluid.

INTRODUCTION

Ofloxacin [(±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]1,4-benzoxazine-6-carboxylic acid (WHO)] (Fig. 1) is a new antibiotic of the DNA-gyrase-inhibitor type. The potent bactericidal effect results from interference with the topoisomerase II of the bacteria [1]. Ofloxacin is active against a wide spectrum of germs on both the gram-positive and the gram-negative sides, including *Pseudomonas aerugi-*

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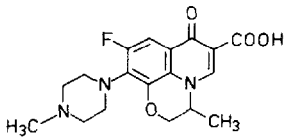


Fig. 1. Chemical structure of ofloxacin.

nosa. Furthermore, a relevant activity against *Mycoplasma* and *Chlamydia* was reported [1].

Microbiological assays of ofloxacin (agar diffusion test) were described by several authors [2–4]. These methods have the disadvantage of showing only a poor limit of detection and of lacking the possibility to differentiate between ofloxacin and possible metabolites or other antibiotics also affecting the test strain used.

Several authors have reported high-performance liquid chromatographic (HPLC) methods for the determination of ofloxacin in biological materials. Groeneveld and Brouwers [5] suggested an extraction assay comprising the addition of an internal standard and UV monitoring at 294 nm. Carlucci et al. [6] reported an anion-exchange assay with a three-step methylene chloride extraction and subsequent back-extraction of ofloxacin into sodium hydroxide solution, resulting in extraction yields between 53 and 66%. This method also made use of UV detection and required the addition of an internal standard. Several authors [7–9] suggested very useful HPLC assays consisting of the direct injection of the supernatant of precipitated plasma and fluorescence detection of ofloxacin. White et al. [10] described a modified ciprofloxacin method, although with almost no linearity or precision data. Sudo et al. [11] reported a thin-layer chromatographic (TLC) method using either a radiochromatogram scanner or UV absorption for detection. This assay, however, is useful for screening metabolites only.

With respect to the various shortcomings of assays mentioned above, it was the objective of this study to develop a simple and sensitive TLC method, making use of the excellent fluorescence characteristics of ofloxacin. Apart from this, the aim of further investigations was to find out whether the use of any of the fluorescence enhancement procedures described in the literature [12–15] can lower the limit of detection or improve the precision of the assay. The applicability of this assay to biological material was to be tested with samples drawn from patients suffering from pleural effusion.

EXPERIMENTAL

Chemicals and materials

Ofloxacin was supplied by Hoechst (Frankfurt, F.R.G.). Solvents (analytical grade), chemicals (analytical grade) and TLC plates were obtained from

E. Merck (Darmstadt, F.R.G.). The TLC plates used were precoated with silica gel 60 (layer thickness 0.25 mm, 20 cm × 20 cm) without a fluorescent indicator or concentrating zone. The plates were prewashed in the solvent used for chromatography, subsequently air-dried and stored under normal conditions. N,N-Dimethylformamide (analytical grade) was purchased from Fluka (Buchs, Switzerland).

Instruments

Drug extracts were evaporated to dryness in a Speed Vac concentrator (Bachofer, Reutlingen, F.R.G.). Solutions were applied to the TLC plates with an Autospotter (Desaga, Heidelberg, F.R.G.). The plates were scanned with a KM3 chromatogram spectrophotometer (Carl Zeiss, Oberkochen, F.R.G.), PMQ 3 amplifier (Carl Zeiss) and a Linseis recorder, Type LS (Linseis, Selb, F.R.G.).

Preparation of plasma and pleural fluid standards

For the preparation of plasma and pleural fluid standards, three ofloxacin stock solutions (1, 10, 100 μg/ml in 0.005 *M* sodium hydroxide) were used. The stock solutions were stored at 4 °C and freshly prepared every four weeks. Various amounts of any of the three stock solutions were diluted with blank plasma or pleural fluid[a] to give the required standard concentrations. Reference standards for the determination of extraction yields were prepared by diluting the stock solutions with N,N-dimethylformamide.

Extraction from pleural fluid

Extraction was performed in silicon-impregnated and screw-capped glass centrifuge tubes. To 1.0 ml of pleural fluid, 1 ml of buffer solution (pH 7) and 5.0 ml of methylene chloride were added. After shaking with a mechanical shaker for 10 min, the tubes were centrifuged (30 min, 4500 *g*, 10 °C) to separate the phases. The aqueous layer was discarded, and 3.0 ml of the organic layer were transferred to another silicon-impregnated centrifuge tube and evaporated to dryness in a Speed Vac concentrator at 60 °C.

Extraction from plasma

Plasma samples were treated in the same way as pleural fluid samples.

Thin-layer chromatography

The dry residue obtained above was dissolved in 100 μl of N,N-dimethylformamide–methanol (9:1, v/v) by shaking on a Heidolph mixer (30 s) and subsequent sonication (3 min). An aliquot (30 μl or smaller volume for

[a]For experimental investigations, diluted plasma (2:1 with water) was substituted for pleural fluid.

samples containing very high ofloxacin levels) of the redissolved extracts was then applied to the TLC plate using the Autospotter (10 mm between spots). The spots were air-dried, and the plate was subsequently developed in an unlined glass tank (Desaga) with ethanol–water (85:5, v/v). The tank also contained two small beakers filled with 30 ml of concentrated ammonia. The equilibration time to saturate the tank atmosphere was 1 h. TLC plates were developed over a distance of 10 cm and finally air-dried.

Fluorescence enhancement

The fluorescence intensity could be enhanced by spraying the dry TLC plate with a solution of citric acid (5% in anhydrous ethanol, modified from ref. 13). The TLC plate was redried, then dipped into liquid paraffin–*n*-hexane (30:70, v/v, modified from ref. 14). Alternatively, a single-step procedure could also be used: for this purpose the plate was quickly dipped into a mixture of 15% paraffin–*n*-hexane and 10% citric acid–anhydrous ethanol (92:8, v/v). The mixture had to be stirred for a few minutes prior to use to remove a slight sedimentation of paraffin.

Furthermore, tests were carried out with the poly(ethylene glycol) derivative Triton X-100 (1:99 in *n*-hexane, v/v) suggested by Ho et al. [15] for the fluorescence enhancement of polycyclic aromatic hydrocarbons.

In order to avoid some loss of precision, the procedures for fluorescence enhancement have to be adhered to most carefully. Spraying should be carried out at intervals to prevent wetting of the plate. For the same reason, plates should be dipped very quickly to preclude spreading of the spots.

Densitometric measurement of the chromatograms

Following chromatography, fluorescence spectra of ofloxacin were scanned manually before and after fluorescence enhancement using the KM3 chromatogram spectrophotometer. Excitation spectra were measured using a deuterium lamp (H 30 DS), an adjustable monochromator and a 390 nm cut-off filter. Emission spectra were determined with the medium-pressure mercury lamp ST 41 (excitation, 313 nm line) and an adjustable monochromator.

For densitometric measurement of the chromatograms, the spectrophotometer was operated in the fluorescence mode (arrangement, monochromator–sample). For a determination of the fluorescence intensity, the 313 nm line of the medium-pressure mercury lamp ST 41 was selected as the excitation wavelength. The emission light was filtered with a 390 nm cut-off filter (slit width 1×6 mm).

Calculation of unknown concentrations

Ofloxacin concentrations (x) were calculated with the linear equation $y = mx + b$ (y=peak area). The slope (m) and intercept (b) of the linear

regression curves (peak areas versus ofloxacin standards) were computed by the least-squares method.

In order to establish a linear regression curve, four plasma or pleural fluid standards (two different concentrations, $n=2$), treated in the same way as the unknown samples, were applied to each TLC plate.

Application of the method

The applicability of the method was demonstrated with ofloxacin samples drawn from two patients suffering from pleural effusion. Each patient had received twice-daily doses of 200 mg of ofloxacin. Plasma samples were drawn prior to and 0.5, 1, 2, 4, 8, 12 and 24 h after drug administration. Samples of pleural fluid were collected at corresponding times from a pleural catheter, applied into the pleural cleft to drain the pleural effusion (the 24-h pleural fluid sample from patient 2 was missing). All clinical samples were stored at −20°C until analysis.

RESULTS

Chromatography

Under the conditions described, ofloxacin showed an R_F value of 0.42. No interference from endogenous material from either plasma or pleural fluid was observed. Representative chromatograms of drug-free and spiked plasma samples are given in Fig. 2. Fig. 3 shows chromatograms of blank and spiked pleural fluid.

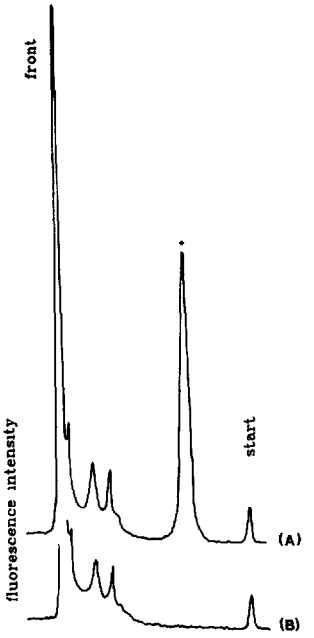
Fluorescence enhancement and lower limit of detection

Spraying the plate with a solution of citric acid produced a 2.3-fold increase in fluorescence intensity. Subsequent dipping in paraffin–*n*-hexane brought about an additional enhancement by a factor of 1.3 (total 3.1).

Dipping an untreated plate into the paraffin solution enhanced fluorescence 4.6-fold. The application of the combined mixture of citric acid and paraffin (single-step method) produced the strongest effect: the fluorescence was enhanced by a factor of 6.7. The use of Triton X-100 had no effect on the fluorescence intensity of ofloxacin.

When the chromatogram was treated by the single-step method, the lower limit of detection was 1 ng/ml in both pleural fluid and plasma samples. This method resulted in coefficients of variation (C.V.) for the measured fluorescence intensities on the plate before and after treating of 8.8% and 4.2%, respectively (ofloxacin extracted from plasma, 120 ng per spot, $n=6$).

Furthermore, the fluorescence intensity of other, partially interfering peaks was not increased to the same extent as that of ofloxacin. This selective fluorescence enhancement improved the signal-to-noise ratio.



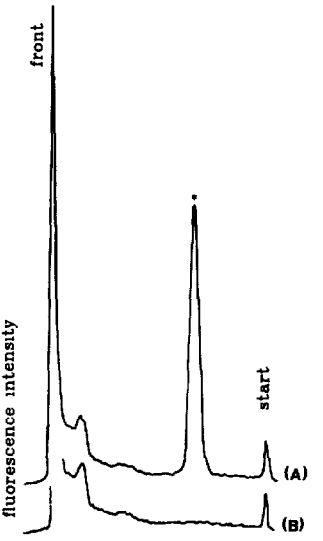


Fig. 2. Representative chromatograms of blank plasma (B) and a plasma sample containing 68.7 ng/ml ofloxacin (A).

Fig. 3. Representative chromatograms of blank pleural fluid (B) and a pleural fluid sample containing 130.0 ng/ml ofloxacin (A).

Fluorescence properties of ofloxacin

The fluorescence properties of untreated and treated ofloxacin spots were determined after development of the TLC plate. For untreated spots the excitation and emission maxima were 296 and 492 nm, respectively. Irrespective of the fluorescence enhancement procedure used, treatment of the plate after chromatography had hardly any effect on the excitation wavelength (maximum 299 nm), whereas the emission maximum was changed to a greater extent, shifting from 492 to 480 nm (± 2 nm). Similar results were reported in a norfloxacin method [12].

Recovery

Recovery studies were performed by extracting and analysing spiked plasma and pleural fluid. For the calculation of extraction yields, the resulting peak areas were compared with those of ofloxacin reference standards in N,N-dimethylformamide (freshly prepared by diluting a stock solution with N,N-dimethylformamide). Reference standards and extracted ofloxacin standards had to be prepared in the same solvent in order to run the Autospotter properly. Samples containing three or four different concentrations of ofloxacin in plasma

TABLE I

EXTRACTION YIELDS AND PRECISION DATA

Concentration of ofloxacin (μg/ml)	n	C.V. (%)	Extraction yield (%)
Plasma			
0.02	10	3.05	102.1
0.2	10	4.04	99.1
2.0	6	4.00	95.6
Pleural fluid			
0.02	10	4.30	98.1
0.05	10	2.25	99.8
0.25	10	3.38	97.6
2.0	10	4.35	97.3

(pleural fluid) were determined ($n=6$ for 2000 ng/ml plasma standards and $n=10$ for all other concentrations). Extraction yields averaged 98.9% for plasma and 98.2% for pleural fluid. Detailed results are given in Table I.

Linearity

The calibration curves were linear in the range 1–2000 ng/ml. Linearity was tested with samples containing 1, 2, 5, 7, 20, 50, 70, 100, 200, 500, 1000, 1500 and 2000 ng/ml plus blank. C.V. values were 0.9995 for plasma and 0.9993 for pleural fluid.

Reproducibility

Reproducibility experiments were carried out for three plasma and four pleural fluid concentrations of ofloxacin ($n=6$ for 2000 ng/ml plasma standards and $n=10$ for all other concentrations). The results obtained are summarized in Table I. The C.V. values ranged from 3.05 to 4.04% for plasma standards and from 2.25 to 4.35% for pleural fluid.

Drug interferences

The following substances were tested for interference with the chromatographic separation and the fluorimetric determination: acetylcysteine, cephazolin, dopamine, furosemide, neomycin, paracetamol, ranitidine, spironolactone, tramadol. None of these agents interfered with ofloxacin.

Applicability of the method

Ofloxacin plasma and pleural fluid levels determined in samples drawn from two patients are presented in Fig. 4. In both patients the plasma levels rose much faster in the initial phase than the corresponding pleural fluid concen-

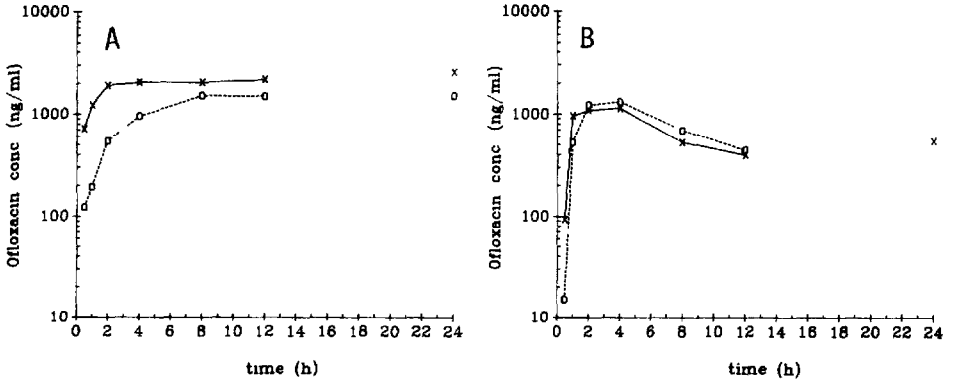


Fig. 4. Plasma (×) and pleural fluid (○) concentration–time curves (semi-logarithmic plot) in two patients after administration of twice-daily doses of 200 mg of ofloxacin.

trations. In patient 1 the ofloxacin levels in pleural fluid and plasma reached similar levels from the eighth hour onwards (Fig. 4A), whereas in patient 2 plasma concentrations were exceeded by pleural fluid levels after 2 h (Fig. 4B).

DISCUSSION

The method described allows a simple and rapid determination of ofloxacin plasma and pleural fluid levels without interference from a large variety of co-administered drugs.

One of the main advantages of this assay is the very low limit of detection, which could be achieved by using various methods of fluorescence enhancement. Both the sensitivity and the precision were increased considerably. These findings are in good agreement with results obtained in the norfloxacin assay [12]. Treating the plate with a mixture of citric acid and paraffin produced the most pronounced increase in fluorescence intensity compared with the increase brought about by either the individual or consecutive use of the two components.

The methods described increase the fluorescence of ofloxacin substantially, and the fluorescence of coextracted substances only moderately. Owing to the high sensitivity of the method, it is possible to perform the analysis with only small sample volumes.

For the determination of the fluorescence intensity, excitation was performed at 313 nm, as this wavelength is the strongest line in the spectrum of the mercury lamp in the proximity of the measured excitation maximum of ofloxacin (299 nm).

As linear results were obtained only in the range 1–2000 ng/ml for plasma or pleural fluid, smaller volumes of the redissolved ofloxacin extracts or diluted

solutions of samples containing higher concentrations of ofloxacin had to be applied to the TLC plates.

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