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QUANTIFICATION OF THE ENANTIOMERS OF OFLOXACIN IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Two methods for the determination of (+)- and (−)-ofloxacin in biological fluids by high-performance liquid chromatography are described. The first method is separation on a chiral stationary phase with bovine serum albumin immobilized on silica gel. The second is the coupling of ofloxacin to L-leucinamide via diphenylphosphinyl chloride activation. The diastereoisomeric derivatives are then separated on a common reversed-phase column. The second method revealed only slight differences in the pharmacokinetics of (+)- and (−)-ofloxacin in humans after an intravenous administration of racemic ofloxacin.

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 (I, Fig. 1), is a totally synthetic, bactericidal drug. Its effect is based on the inhibition of the DNA-gyrase of bacteria. Ofloxacin is marketed as a racemate, i.e. a mixture of the two optical isomers (−)- and (+)-ofloxacin. In vitro trials with different bacterial strains showed that the (−)-isomer is more potent than the (+)-isomer [1]. For determination of the enantiomeric stability in the body and monitoring of the pharmacokinetic behaviour of each enantiometer, it is desirable to determine the two enantiomers simultaneously in biological fluids.

For chromatographic separation of enantiomers it is always necessary to introduce a chiral environment to the analyte. This can be done by various methods, for example by using a chiral stationary phase, by adding a chiral moiety to the mobile phase or by derivatisation of the analyte with a chiral reagent.

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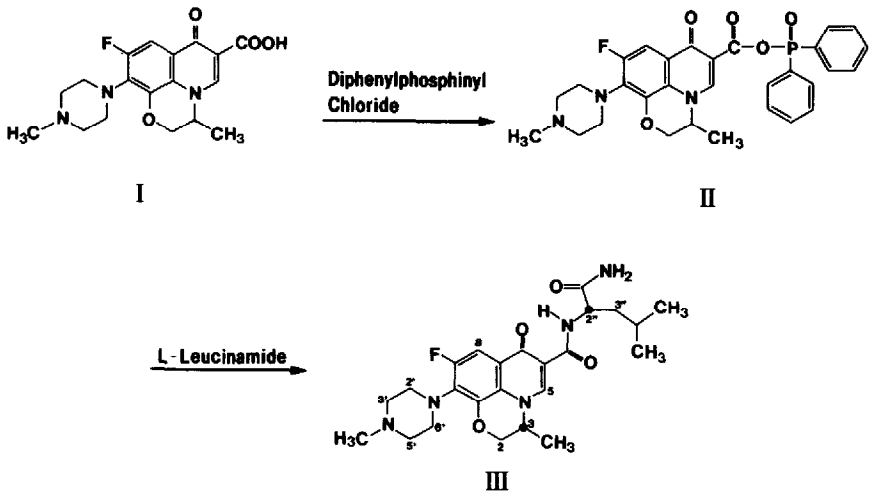

I II III

Fig. 1. Structures of ofloxacin (I), the mixed anhydride (II) and the L-leucinamide derivative (III).

This paper describes two enantiospecific high-performance liquid chromatographic (HPLC) methods for the determination of the ofloxacin enantiomers: direct resolution on bovine serum albumin covalently bonded to wide-pore Nucleosil, and derivatization with the chiral reagent L-leucinamide, followed by separation of the diastereomers formed. The analysis of serum and urine samples from volunteers was carried out using the latter method.

EXPERIMENTAL

Reagents

The following solvents and reagents were used without specific purification: acetonitrile (HPLC grade S, Rathburn, Walkerburn, U.K.), dichloromethane A.R., phosphoric acid A.R., triethylamine pure, exsiccated sodium sulphate and ready-for-use phosphate buffer (pH 7) (Riedel de Haën, Seelze-Hannover, F.R.G.), L-leucinamide hydrochloride (>99%, Aldrich, Milwaukee, WI, U.S.A.), diphenylphosphinyl chloride (Janssen, Beerse, Belgium), tetraethylammonium hydroxide (20% in water) (Fluka, Buchs, Switzerland).

The L-leucinamide solution (ca. 300 mmol/l) was prepared by adding 10 ml of dichloromethane and 1 ml of 5 *M* sodium hydroxide solution to 500 mg of L-leucinamide hydrochloride. After shaking, the upper aqueous layer was aspirated and discarded. The dichloromethane layer was retained and stored over exsiccated sodium sulphate.

Standards

Racemic ofloxacin and >99% pure (−)- and (+)-ofloxacin were supplied by Daiichi Seiyaku (Tokyo, Japan). Stock solutions (1 mg/ml) of ofloxacin (±, −, and +) were prepared by dissolving 10 mg of each compound in 1 ml of 0.2 *M* sodium hydroxide solution followed by the addition of 9 ml of phosphate buffer

(pH 7). The solutions were stable in the dark at 4°C for at least two months. Plasma standards were prepared by adding the corresponding volumes of the stock solutions to the drug-free plasma and urine. The standards were stored frozen at −20°C.

Pure (−)-ofloxacin-L-leucinamide (III, Fig. 1) was synthesized by activating 0.15 mmol of (−)-ofloxacin with 0.3 mmol of triethylamine and 0.15 mmol of diphenylphosphinyl chloride (10 min), followed by the addition of 0.15 mmol of L-leucinamide and subsequent stirring for 1 h. The total reaction was carried out in 25 ml of dichloromethane at room temperature. The dichloromethane and triethylamine were subsequently evaporated, the residue was reconstituted in 0.5 ml of dichloromethane, and (−)-ofloxacin-L-leucinamide was precipitated by the dropwise addition of *n*-hexane.

Work-up for direct resolution on Resolvosil

A 1-ml volume of plasma (serum) or 0.2 ml of urine was mixed with 1 ml of buffer (pH 7) and shaken with 5 ml of dichloromethane. After centrifugation at 2500 *g* for 5 min, the aqueous supernatant was aspirated and discarded. Then 4 ml of the organic phase was transferred to a conical centrifuge tube, and the dichloromethane was evaporated under nitrogen. Finally, the residue was dissolved in 200 µl of the mobile phase.

Work-up and derivatization of plasma and urine samples

A 0.5-ml volume of plasma (serum) or 0.2 ml of urine was mixed with 1 ml of buffer (pH 7) and shaken with 2 ml of dichloromethane. After centrifugation at 2500 *g* for 5 min, the aqueous supernatant was aspirated and discarded. For the derivatization, 1 ml of the dichloromethane extract was added to a polypropylene centrifuge tube containing 1 ml of dichloromethane, 20 µl (0.105 mmol) of diphenylphosphinyl chloride and 20 µl (0.144 mmol) of triethylamine. The mixture was vortexed for 10 s. Then 500 µl of the L-leucinamide solution (ca. 0.150 mmol) were added and the mixture was shaken for 10 min. The mixture obtained from the plasma (serum) sample was extracted with 200 µl of 1 *M* hydrochloric acid and the mixture from urine samples with 500 µl of 0.5 *M* hydrochloric acid. In each case 100 µl of the aqueous supernatant were injected into the HPLC apparatus.

High-performance liquid chromatography

The HPLC system consisted of a 300 B solvent pump (Gynkotek, Germering, F.R.G.), an autosampler BT 7041 (Biotronik, Maintal, F.R.G.), a temperature-control system (Waters, Milford, MA, U.S.A.) and an RF-530 fluorometer (Shimadzu, Düsseldorf, F.R.G.) operated at 298 nm for excitation and 458 nm for emission.

Chromatographic separation of the underivatized ofloxacin enantiomers was achieved on an ET 150/8/4 Resolvosil-BSA-7 (150 mm×4 mm I.D.) analytical column (Macherey-Nagel, Düren, F.R.G.) connected to the corresponding guard column CT 30/6/4. The mobile phase was 0.2 *M* phosphate buffer (pH 8.0)–propan-2-ol (97:3). The chromatograph was operated at ambient temper-

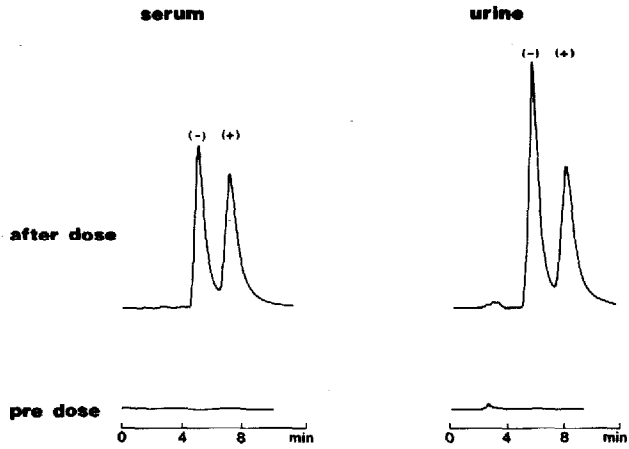

Fig. 2. Chromatograms of extracts from samples obtained from a volunteer. The serum sample was taken 5 h after an oral dose of 400 mg of (±)-ofloxacin and contained 1.135 μg/ml (−)-isomer and 1.065 μg/ml (+)-isomer. The urine sample was taken between 36 and 48 h and contained 7.005 μg/ml (−)-isomer and 4.814 μg/ml (+)-isomer.

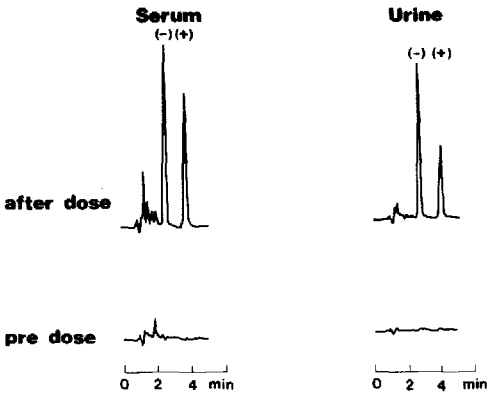

Fig. 3. Chromatograms of derivatized samples from a volunteer. The serum sample was taken 45 min after an intravenous dose of 50 mg of (±)-ofloxacin and contained 0.176 μg/ml (−)-isomer and 0.166 μg/ml (+)-isomer. The urine sample was taken between 36 and 48 h and contained 0.912 μg/ml (−)-isomer and 0.540 μg/ml (+)-isomer.

ature and a flow-rate of 1.0 ml/min. Under these conditions the retention times were ca. 5.2 min for the (−)-enantiomer and 7.5 min for the (+)-enantiomer. Chromatograms of blank and real samples are shown in Fig. 2.

Chromatographic separation of the diastereomeric ofloxacin derivatives was achieved on a 125 mm × 4.6 mm I.D. column filled with Nucleosil 120-5 C_{18}, particle size 5 μm (Macherey-Nagel). The mobile phase was 0.2 *M* phosphoric acid, adjusted to pH 1.85 with tetraethylammonium hydroxide solution, and acetonitrile (80:20). The flow-rate was 1.5-ml/min and column temperature 40°C. Under these conditions, the retention times were ca. 2.6 min for the (−)-derivative and 3.8 min for the (+)-derivative. Chromatograms of blank and real samples are shown in Fig. 3. Quantification was based on the peak area of the substance of interest.

Thin-layer chromatography (TLC)

Separation was performed on silica gel F_{254} pre-coated plates for nano-TLC (No. 5642, E. Merck, Darmstadt, F.R.G.) in a twin-trough chamber (10 cm×10 cm) with chamber saturation. The mobile phase was chloroform–methanol–triethylamine (3:1:1). The developing time was 25 min.

Mass spectrometry

The mass spectra were recorded with the MS 80-RFA mass spectrometer (Kratos, Manchester, U.K.).

^{1}H NMR spectroscopy

^{1}H NMR spectra were measured at 270 MHz in [^{2}H]trichloromethane solution with tetramethyl silane as standard using a Bruker AM-270 instrument.

RESULTS

Chromatography on Resolvosil

Resolvosil is a chiral stationary phase based on bovine serum albumin, covalently bonded to wide-pore Nucleosil. The separation of the ofloxacin enantiomers was achieved by using the optimum pH and buffer concentration values from the proposed range in the literature [2]. A mobile phase with a 0.2 *M* phosphate buffer (pH 8.0) and 3% propan-2-ol, added as a cosolvent, gave the best results. The resolution was ca. 0.7.

The assignment of the peaks was unambiguous since the pure (+)- and (−)-enantiomers were available. This chromatographic system is also suitable for the separation of the ofloxacin enantiomers extracted from serum and urine. As can be seen from Fig. 2, there are no interferences present.

Derivatization method

The diastereomeric L-leucinamide derivatives of (−)- and (+)-ofloxacin are resolved by common reversed-phase HPLC and also by normal-phase high-performance thin-layer chromatography (R_F values: (−), 0.57; (+), 0.49). In HPLC the k' value is 2.6 for the (−)- and 3.8 for the (+)- derivative. These values give a separation factor (α) of 1.5. The resolution (R_s) is greater than 3. This separation enables traces of one enantiomer to be determined against a bulk amount of the other.

Assay validation

For assay validation, (±)-ofloxacin was mixed over the concentration range 0.03–10 μg/ml with human blank plasma and 0.3–100 μg/ml with urine. In addition, admixtures of 1 μg of (−)-ofloxacin per ml of plasma were made up with 0, 2 and 5% of the (+)-enantiomer. Corresponding admixtures containing urine in place of the plasma (10 μg of ofloxacin per ml of urine) were also made up.

Each mixture was divided into several portions. Extractions and chromatography were carried out in two series on two separate days. These data provided

TABLE I

STATISTICAL VALIDATION OF THE HPLC ASSAY OF PLASMA SAMPLES

Amount added (μg/ml)		Amount found (μg/ml)		n	Coefficient of variation (%)	
(−)-Isomer	(+)-Isomer	(−)-Isomer	(+)-Isomer		(−)-Isomer	(+)-Isomer
0.015	0.015	0.017 ± 0.002	0.015 ± 0.002	7	12.6	12.9
0.150	0.150	0.161 ± 0.016	0.159 ± 0.016	7	9.8	9.7
5.000	5.000	5.112 ± 0.360	5.264 ± 0.256	7	7.1	4.9
1.000	0.000	1.058 ± 0.056	0.004 ± 0.001	7	5.3	25.0
1.000	0.020	1.031 ± 0.067	0.023 ± 0.001	7	6.5	6.1
1.000	0.050	1.009 ± 0.079	0.053 ± 0.004	7	7.8	6.9

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY OF URINE SAMPLES

Amount added (μg/ml)		Amount found (μg/ml)		n	Coefficient of variation (%)	
(−)-Isomer	(+)-Isomer	(−)-Isomer	(+)-Isomer		(−)-Isomer	(+)-Isomer
0.150	0.150	0.149 ± 0.028	0.156 ± 0.025	7	18.8	16.1
1.500	1.500	1.466 ± 0.071	1.453 ± 0.082	7	4.9	5.6
50.000	50.000	50.400 ± 1.454	50.571 ± 1.449	7	2.9	2.9
10.000	0.000	10.129 ± 0.974	0.000	7	9.6	–
10.000	0.200	9.829 ± 0.966	0.214 ± 0.032	7	9.8	14.7
10.000	0.500	9.786 ± 0.871	0.507 ± 0.043	7	8.9	8.5

us with a measure of accuracy, linearity and precision of the assay (Tables I and II).

Derivatization and determination of an admixture of (−)-ofloxacin in a blank plasma sample (Table I) resulted in 0.4% (+)-enantiomer. Whether this small amount is due to racemization during derivatization, or to enantiomeric impurity in (−)-ofloxacin or L-leucinamide is questionable. Nevertheless, no relevant racemization takes place during derivatization. In the analogous urine sample (Table II), the same percentage of (+)-enantiomer is below the detection limit and thus no (+)-enantiomer is found.

Large amounts of the one enantiomer do not interfere with the determination of the other. This is verified by the ability to determine the 0, 2 and 5% admixtures of the (+)-enantiomer against the (−)-enantiomer (Tables I and II).

Detection limit

The detection limits corresponding to three times the standard deviation for the lowest amount measured are 3 ng/ml for plasma and 80 ng/ml for urine.

Extraction and reaction yield

The overall yield of the sample preparation, i.e. the extraction yield of ofloxacin from plasma, the reaction yield of the derivatization step and the extraction yield of the derivative in acidic upper aqueous layer, was 65%.

Mass and ^1H NMR spectra of the ofloxacin derivative

The isobutane ionization mass spectra confirmed the structure of the ofloxacin-leucinamide derivative. The pseudomolecular ion $(M+H)^+$ appeared as expected at *m/z* 474 and was accompanied by $(M-NH_2)^+$ at 457 and by $(M-CONH_2)^+$ at 429.

The ^1H NMR spectrum also confirms the structure (III, Fig. 1): $\delta = 10.36$ (N-H), 8.61 (5-H), 7.73 (8-H), 6.45 (N-H), 5.31 (N-H), 4.2–4.7 (2,3,2″-H), 3.40 (2′,6′-H), 2.60 (3′,5′-H), 2.40 (N-CH_3), 1.58 (3-CH_3), 1.24 (3″-H), 0.9–1.0 (2×CH_3).

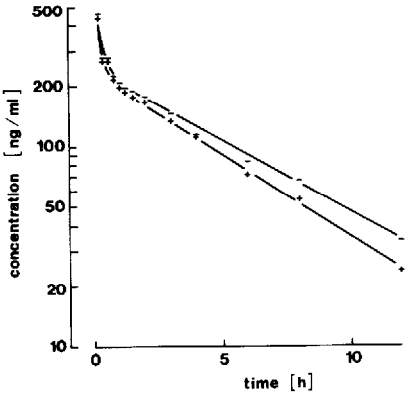

Fig. 4. Serum concentration versus time curve of the ofloxacin enantiomers after a single 50-mg intravenous dose of racemic ofloxacin in a volunteer. Curves: – = (–)-isomer; + = (+)-isomer.

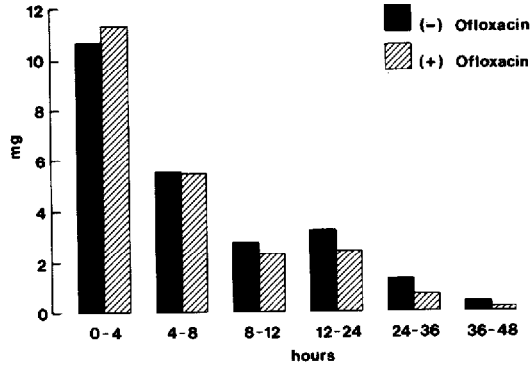

Fig. 5. Renal excretion of ofloxacin enantiomers after a single 50-mg intravenous dose of racemic ofloxacin.

Pharmacokinetics of (+)- and (−)-ofloxacin in a volunteer

This method was utilized in the analysis of serum and urine samples from volunteers dosed with ofloxacin. Fig. 4, for example, shows the concentration curves of the ofloxacin enantiomers after intravenous administration of 50 mg of racemic ofloxacin. There are slightly higher levels of the active (−)-isomer. For the same volunteer there was also a difference in the renal excretion of the enantiomers (Fig. 5). Initially, during the first hours, the excretion of the (+)-isomer was marginally higher, but later the excretion of the (−)-isomer was higher.

DISCUSSION

Each of the two separation methods described here has its strengths and weaknesses. The separation on the chiral column is more attractive, in principle, and the preparation of the samples simpler. The chiral derivatization technique can be carried out on common HPLC columns that have a long life-time and a large number of theoretical plates. The latter derivatization method was chosen for the following reasons: better resolution enabling us to determine trace amounts of (+)-ofloxacin against a bulk of (−)-ofloxacin, and the ability to measure a large series of samples with one column.

Derivatization method

The quick and complete derivatization of carboxylic acids with amines can only be achieved via activated intermediates, e.g. acid chlorides or mixed anhydrides. Reagents such as alkyl chloroformates [3] and carbodiimides [4] used with other carboxylic acids produced incomplete reactions or rearrangements on reaction with ofloxacin. This is probably because ofloxacin is an unsaturated carboxylic acid with limited carbonyl activity. Diphenylphosphonic chloride is the reagent recommended for activating 2-alkenoic acids for amide formation [5].

Using this reagent, a rapid reaction takes place at room temperature giving the mixed anhydride II (Fig. 1), which in turn reacts with L-leucinamide to produce pure (−)-ofloxacin-L-leucinamide (III). The compound was verified by mass and ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum also indicated that no attack had taken place at the β-carbon of the conjugated system.

Chromatographic resolution of diastereomers

At first glance, the significantly different retention times in the chromatography of these diastereomeric derivatives are very surprising, especially if one considers that the two chiral carbon atoms are separated by six bonds, three of which are not part of the planar ofloxacin ring system. However, intramolecular hydrogen bonds probably have a considerable influence and could fix the leucinamide part of the molecule in the same plane as ofloxacin. This hypothesis is supported by the fact that we could not separate the corresponding diastereomeric (*S*)-α-(1-naphthyl)ethylamine, L-1-phenylethylamine and D-1-phenylethylamine derivatives, which cannot form these hydrogen bonds.

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