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An HPLC assay and a microbiological assay to determine levofloxacin in soft tissue, bone, bile and serum

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Abstract

A simple, specific and sensitive HPLC assay for levofloxacin in serum, bile, soft tissue and bone was evaluated and validated. The samples were prepared by protein precipitation with acids and methanol, which yielded high recoveries (for serum and bile > 98% and for bone and soft tissue > 90%). The compounds were separated on a reversed phase column with an acidic mobile phase containing triethylamine. The eluate was monitored by fluorescence detection. The HPLC assay is linear over the usable concentration range (0.1–40 μg/ml) and it provides good validation data for accuracy and precision. Although comparison of HPLC results to the results of a microbiological assay showed congruent results (regression coefficients > 0.967). HPLC should be the method of choice for determination of levofloxacin in biological matrices.

Keywords: Levofloxacin; Reversed-phase chromatography; Microbiological assay; Human tissues; Bile

1. Introduction

Levofloxacin, full name (s)-(-)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, is a new quinolone antibacterial agent and the S (-) enantiomer of the racemate ofloxacin. In the literature much information about ofloxacin levels in serum and tissue exists [1] however, there is little information concerning tissue concentrations after dosing of 500 mg levofloxacin. Since the target sites of antibiotics are predominantly located in tissues, adequate tissue penetration is mandatory.

To determine levofloxacin and oflocaxin in tissue and biological fluids HPLC assays and microbiological assays are used. Numerous analytical methods have been published which describe the HPLC assay for quinolones in biological fluids [2–6]. For bone and tissue, only few of the published methods give exact validation data [7–10].

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In this paper a simple reversed phase HPLC method with excellent specificity, linearity and accuracy for levofloxacin is described and compared to a microbiological assay. Advantages and disadvantages of both methods as well as their application for the analysis of different biological matrices are discussed.

2. Experimental

2.1. Material

Levofloxacin and its main metabolites, desmethyl-levofloxacin and levofloxacin-N-oxide were obtained from Aventis Pharmaceuticals (Frankfurt, Germany). The internal standard ciprofloxacin was a gift from Bayer Pharmaceuticals (Leverkusen, Germany).

The mobile phase contained gradient grade methanol, phosphoric acid and triethylamine from Merck (Darmstadt, Germany) and bi-distilled water. A Waters Symmetry C_{18} 5 μm, 150 × 4.6 mm I.D. column (Eschborn, Germany) was used.

IsoSensitest agar was purchased from Oxoid LTD (Hampshire, UK).

2.2. Instrumentation

HPLC-analysis was performed using a Perkin Elmer series 200 LC pump, ISS 200 autosampler and LC 240 fluorescence detector (Überlingen, Germany). Data integration and manipulation were performed using Turbochrom-Software (Überlingen, Germany).

2.3. HPLC conditions

Levofloxacin, its desmethyl-metabolite and the internal standard were eluted isocratically with a flow rate of 1.5 ml/min using a mobile phase consisting of water, methanol and triethylamine (750:250:4 V/V/V) adjusted to pH 3 with orthophosphoric acid. The mobile phase was degassed using a vacuum degasser Series 200 Perkin Elmer (Überlingen, Germany). Elution of levofloxacin-N-oxide required a higher concentration of methanol. After 10 min of isocratic elution a linear gradient was run for 5 min adding 20% methanol (600:400:4 V/V/V).

The wavelengths of the fluorescence detector were set to 295 (excitation) and 490 nm (emission), respectively.

2.4. Sample preparation

2.4.1. Serum/bile

All samples were stored at −30°C until analysis. 200 μl aliquots of serum were mixed with 20 μl internal standard solution (100 μg/ml), 200 μl water and 800 μl methanol. Bile samples were prepared likewise, except that 40 μl internal standard solution were added, because higher concentrations were expected. Calibration standards were prepared the same way using 200 μl aliquots from aqueous solutions containing 0.1, 0.5, 1, 2, 4, 6, 8, 10, 14, 16, 20, 30, and 40 μg/ml levofloxacin. After incubation for 20 min at 25°C, the precipitated protein was separated via centrifugation (15 min/15 000 × g). A total of 20 μl of the supernatant was injected into the HPLC apparatus.

2.4.2. Bone/soft tissue

The bone fragments (deep frozen in liquid nitrogen) were crushed into very small pieces with a mortar and a pestle. Soft tissue was dissected into 20–30 mg pieces. A total of 200 mg of the bone fragments or of soft tissue, 50 μl internal standard solution and 2 ml homogenisation reagent consisting of water, methanol, perchloric acid 70% and phosphoric acid (500: 500: 10: 1 V/V/V/V) were placed into 4 ml tubes. After 15 min, the suspension was homogenised using an Ultra Turrax for 1 min. To ensure complete extraction of levofloxacin, the homogenised samples were stored overnight at 4°C. After centrifugation (5 min/4000 × g), 20 μl of the supernatant was injected.

Calibration standards for bone and soft tissue were prepared by mixing: 200 μl of aqueous stock solutions containing 0.1, 0.5, 1, 2, 4, 6, 8, 10, 14, 16, 20, 30, and 40 μg/ml levofloxacin, 20 μl internal standard and 1 ml homogenisation reagent were mixed.

2.5. Microbiological assay

Samples for the microbiological assay were prepared as already described. A sterile buffer, containing K_2HPO_4 13.6 g and KH_2PO_4 4.0 g of 1 l water adjusted at pH 7, was used instead of water/methanol or homogenisation reagent. Calibration solutions were prepared with buffer too. The assay plates (25×25 cm) contained 140 ml IsoSensitest agar inoculated with 0.3 ml of an overnight culture of *Escherichia coli* (ATCC 35218). 9 mm diameter wells were punched into the agar and filled with either 100 µl sample supernatant or calibration solution. After the plates were kept for 1 h at room temperature, they were incubated overnight at 37°C. The inhibition zones around the wells were measured with a Behringwerke modified immunoassay projector (Marburg, Germany). Unknown concentrations were calculated from the calibration curve constructed using Bennett's calculation [11,12].

3. Results and discussion

3.1. Sample preparation and recovery

Percentage recovery was determined by comparing the peak area ratios of the chromatograms obtained from serum or tissue spiked with known amounts of levofloxacin to those of aqueous standard solutions.

Recoveries of the target analyte levofloxacin from serum, bile and soft tissue and bone are shown in Table 1. In this study, recoveries of $\sim 100\%$ were achieved at all concentrations in serum and bile. Likewise recoveries of $> 90\%$ were consistently achieved in bone and soft tissue.

In the literature recoveries for quinolones in biological fluids often $> 95\%$ are given [2,3,13]. In tissue and bone, recoveries between 70 and 110% are reported [7,8,10,13,14].

4. Chromatography

4.1. Specificity

At a flow rate of 1.5 ml/min, the retention times were as follows: levofloxacin 4.4 min, desmethyl-levofloxacin 5.3 min, ciprofloxacin 6.4 min and levofloxacin-N-oxide 10.7 min. Typical chromatograms obtained from serum and bile are shown in Fig. 1, similar chromatograms were obtained from bone or tissue samples.

Specificity was demonstrated by measuring blank serum and tissue samples, or serum and tissue samples spiked with levofloxacin and ciprofloxacin, desmethyl-levofloxacin and levofloxacin-N-oxide. No interfering peaks were

Table 1
Recoveries of levofloxacin and its metabolites from different matrices

Type of sample	*n*	Concentration added (µg/ml)	Concentration found (µg/ml)	Rel. SD (%) interday	Bias (%)
Serum	8	1	0.97	5.1	−0.7
Serum	8	2	2.03	5.3	+1.5
Serum	8	4	4.11	4.8	+2,8
Serum	8	6	6.28	4.02	+4.7
Serum	8	10	10.18	4.6	−1.8
Bile	6	10	10.06	0.6	+0.6
Bile	6	20	20.14	1.2	+0.7
Bone	4	2	2.01	10.7	+0.5
Bone	4	4	3.67	3.1	−8.9
Bone	4	8	7.46	3.9	−6.7
Soft tissue	4	2	2,21	2.1	+10.6
Soft tissue	4	4	4.15	3.4	+3.8
Soft tissue	4	8	8.27	6.5	+3.4

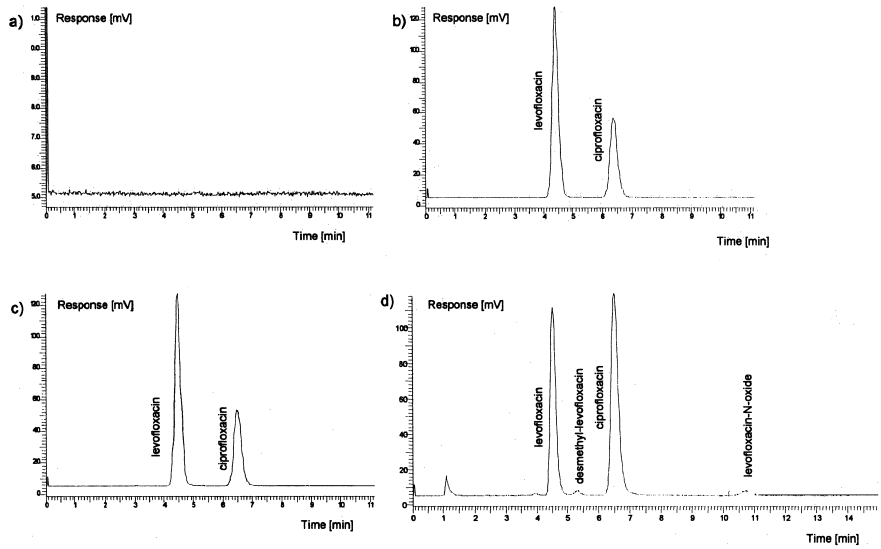

Fig. 1. Chromatograms: (a) drug free serum; (b) patient serum containing ~6.1 μg/ml levofloxacin; (c) control serum spiked with ISD and 6 μg/ml levofloxacin; (d) patients bile containing levofloxacin (5.4 μg/ml) and metabolites desmethyl-levofloxacin (0.2 μg/ml) and levofloxacin-N-oxide (0.3 μg/ml), 40 μl ISD solution were added to bile samples.

observed in blank serum, bile or tissue. The assay is non-enatiospecific.

4.2. Linearity

Calibration curves were constructed in the range of expected concentrations (0.1–40 μg/ml in serum). Linearity was shown using a regression analysis of calibration curves. The regression analysis of the calibration curves of levofloxacin generated a line with a correlation coefficient of >0.998. Thus, the assay is linear for levofloxacin over the usable concentration range.

4.3. Sensitivity

The results of our study indicate that it is possible to detect levofloxacin, desmethyl-levofloxacin and levofloxacin-N-oxide (signal-to-noise ratio 3:1) at a concentration of 1 ng/ml in an aqueous solution. In the course of the clean up samples were diluted ~10-fold, i.e. in serum and tissue the detection limit was 10 ng/ml for levofloxacin.

4.4. Precision

Reproducibility was assessed by performing replicate analyses of different samples. Drug free sera were spiked on 8 days with different concentrations of levofloxacin. The standard deviations are presented in Table 1.

Six serum samples from patients, who got 500 mg levofloxacin orally, were analysed on 3 different days over 2 months and standard deviations were determined. The interday coefficients of variations ranged from 2.5 to 4.9%. The mean intraday coefficient of variations was 1.58% (range 0–1.96%).

4.5. Accuracy and external quality assurance

As shown in Table 1, the overall bias in serum ranged from −0.7% to +4.7%. In bile the overall bias ranged from +0.6 to +0.7%, from −8.9 to +0.5% in bone and from +3.4 to +10.6% in soft tissue.

Six samples of cortical bone, six samples of cancellous bone and three samples of soft tissue from one patient, who received 500 mg levofloxacin iv were prepared and assayed, the

mean and the standard deviation was calculated. Samples of the same tissue were sent to another laboratory (Kees, Department of Pharmacology, University of Regensburg, Germany) and analysed using a similar HPLC-method.

The difference between the inter laboratory means was calculated as 8.2, 5.9 and 4.0% for soft tissue, cortical and cancellous bone, respectively (Table 2). For inhomogeneous samples such as soft tissue and bone that present technical problems including blood contamination and variable moisture content, a standard deviation of 20% was considered to be acceptable [15].

The effect of blood contamination is a topic of special interest. For certain antibiotics (i.e. aminoglycosides and some cephalosporins) that demonstrate a low volume of distribution and a large difference between blood and tissue concentration, a correction for the blood content is usually necessary [16,17]. If an antimicrobial agent is evenly distributed between tissue cells, plasma and red blood cells, the presence of blood will have only a minor effect. That appears to be the case for the quinolones.

In this investigation a blood contamination between 5 and 14% was determined applying the hexacyanoferrat method (Roche Diagnostics, Mannheim, Germany), comparable to the values estimated by Plaue [18]. Hence it should follow a correction of $\sim 20\%$. As described the relative standard deviation for bone is $\sim 20\%$ as well. Thus, a correction of the bone concentration was deemed unnecessary.

4.6. Correlation between HPLC method and microbiological assay

In 19 tissue and 18 bile samples, the levofloxacin level was confirmed by a microbiological assay. Every sample was measured three times with each assay. Correlation coefficients of the microbiological assay's calibration line were > 0.985, for HPLC assay > 0.998. Standard deviations for calibration curves and samples were higher for the microbiological assay than for the HPLC assay. A good correlation was achieved, as coefficients of 0.967 for tissue samples and 0.959 for bile samples between both assays were observed (Fig. 2). By comparing the microbiological assay with the HPLC assay for quinolones in serum, Griggs et. al achieved a coefficient of 0.956, while other authors reported coefficients between 0.845 and 0.555 [5,19,20]. The slope was ~ 1 for the tissue samples. For bile samples,

Table 2
Comparison of two laboratory's results

Sample	Concentration (μg/g) RE[a]	Mean RE	Concentration (μg/g) HD[b]	Mean and (standard deviation) HD	Difference between inter lab. means
Soft tissue	8.43		8.17	7.9	
	8.91		7.93		
		8.67	7.79	(2.4%)	8.2%
Cortical bone	1.31		0.99		
	1.06		1.28		
			1.52		
			0.92	1.3	
			1.67		
		1.18	1.16	(23.6%)	5.9%
Cancellous bone	5.22		7.06		
	6.25		7.04		
			6.05		
			5.01	5.9	
			5.45		
		5.74	5.23	(15.13%)	4.0%

[a] RE, laboratory in Regensburg Germany.
[b] HD, own laboratory in Heidelberg.

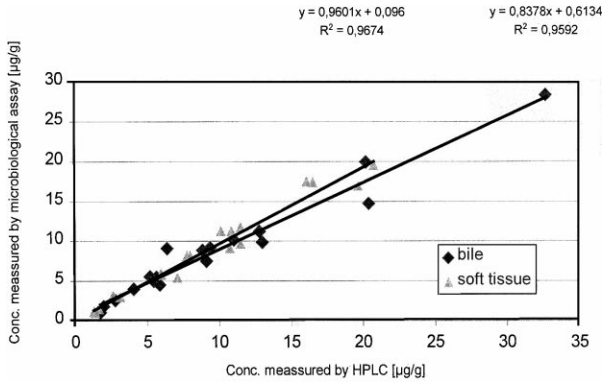

Fig. 2. Correlation between microbiological assay and HPLC. The legend is given in the figure.

however, the slope was 0.84 (i.e. values estimated using HLPC in bile fluid are ~15% higher than those obtained with the microbiological assay). This result seems noteworthy. Possible mechanisms could be: (1) the interference of the pH on either the assay or on the activity of levofloxacin; or (2) or the generation of complexes consisting of contents of bile and levofloxacin thus resulting in a diffusion barrier. A negative effect of the pH on the activity of levofloxacin has been excluded [21].

5. Application

The HPLC assay was applied to pharmacokinetic studies. The study protocols were approved by the local ethic committee, and written consent was obtained from each volunteer.

In 10 patients with biliary obstruction, a nasobiliary tube was placed endoscopically. Each patient received two oral doses of 500 mg levofloxacin (0:24 h). Blood and bile samples were taken at 0, 3, 5, 24, 27, and 29 h postdosing. The typical course of bile and serum is presented for two patients in Fig. 3.

6. Conclusion

The HPLC assay described here, a modification of the method described by Kees et al. [8], uses a single step sample preparation and demonstrate acceptable validation data with a high specificity for levofloxacin. It can be used for the determination of levofloxacin in serum, bile and bone and a variety of human tissues such as granulation tissue, skin, fatty tissue and lung. Ciprofloxacin, desmethyl-levofloxacin and levofloxacin-N-oxide can be detected in these matrices as well. Insofar as the metabolites appeared to be present in negligible amounts, it would appear that routine testing for levofloxacin alone can be conducted isocratically within 10 min.

On comparing the microbiological assay and the HPLC assay, the microbiological assay is found to be less expensive. Disadvantages, of the microbiological assay are the possible interference of other antibiotics and active metabolites, technical difficulties in handling living bacteria and the measuring of the diffuse inhibition zones. Advan-

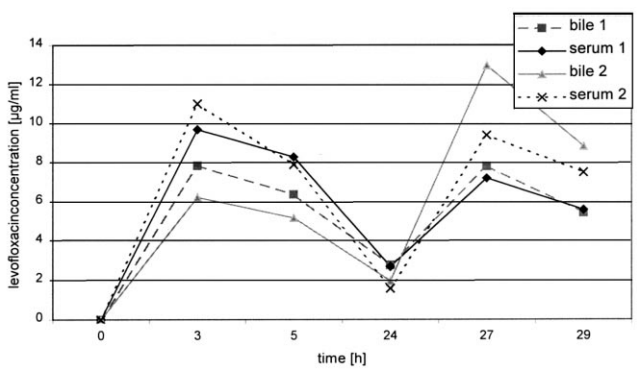

Fig. 3. Serum and bile concentrations of two patients after administration of two oral doses of levofloxacin. Concentrations for bile and serum for patient 1 and 2 as given in the legend of the figure.

tages of the HPLC method are its excellent precision and specificity.

For determination of quinolone levels in human matrices, the HPLC assay should be the method of choice, although under optimised conditions, the microbiological assay may yield comparable results.

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