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Journal of Chromatography B, 700 (1997) 147–153

JOURNAL OF CHROMATOGRAPHY B

Simultaneous quantification of cefotaxime, desacetylcefotaxime, ofloxacine and ciprofloxacine in ocular aqueous humor and in plasma by high-performance liquid chromatography

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Received 7 February 1997; received in revised form 27 May 1997; accepted 2 June 1997

Abstract

Cefotaxime, given intravenously, is currently used as a broad-spectrum antibiotic for prophylaxis of intra- and postoperative infections in ocular lens surgery. A proposed therapeutic and economic alternative is the use of orally active fluoroquinolone ofloxacine as prophylactic agent. A HPLC method was developed for determination of both antibiotics in ocular aqueous humor and plasma in order to optimize dosage for safe surpassing minimal inhibitory concentration in the humor compartment. For plasma determinations a solid-phase extraction procedure was used with ciprofloxacine as internal standard. Detection limits for direct HPLC-analysis of ocular aqueous humor was 0.08 μg/ml for all compounds, whereas in plasma 0.31 μg/ml could be determined after solid-phase extraction. © 1997 Elsevier Science B.V.

Keywords: Cefotaxime; Desacetylcefotaxime; Ofloxacine; Ciprofloxacine

1. Introduction

Methods for pharmacokinetic studies of cefotaxime and ofloxacine were yet readily available. Most of them rely upon HPLC combined with liquid phase extractions [1–24]. None of them allows simultaneous determination of both compounds. Our goal of comparing the distribution of both antibiotics in the compartment of ocular aqueous humor for optimisation of dosage and application time requires this possibility due to the scarce amount of specimen volume. Therefore we developed a high-performance liquid chromatography-based method for quantification of cefotaxime, its main active metabolite desacetylcefotaxime and ofloxacine in plasma after solid-phase extraction of the analytes, and for direct determination in ocular aqueous humor. Internal standardization with ciprofloxacine was used to stabilize precision of the analysis, a compound that may be of interest too in pharmacokinetic studies. The chemical structures of the analytes are given in Fig. 1.

2. Materials and methods

2.1. Chemicals

Cefotaxime, sodium salt, anti-cefotaxime, desacetylcefotaxime, sodium salt and desacetylcefotaxime-

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[1]This manuscript includes portions of a doctoral thesis by R. Gehrke.

0378-4347/97/$17.00
PII S0378-4347(97)00323-X

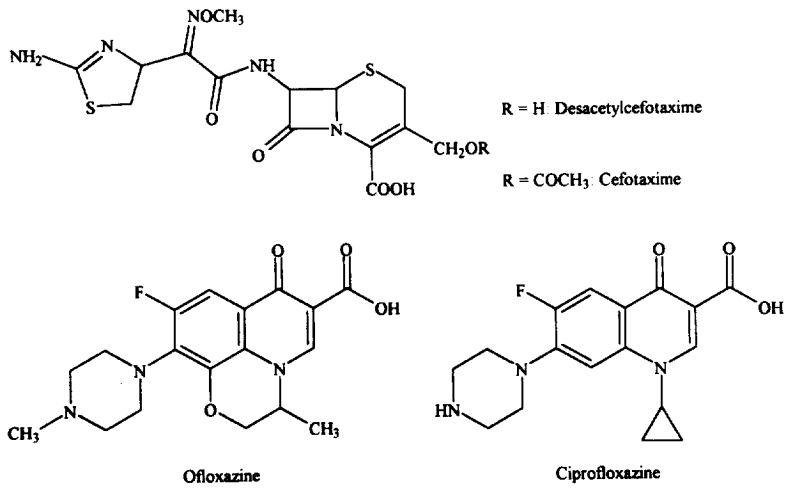

Fig. 1. Chemical structures of the analytes.

lactone were generous gifts from Hoechst (Frankfurt/Main, Germany). Ciprofloxacine was kindly donated by Bayer (Leverkusen, Germany). Ofloxacine was also a kind gift of Daiichi Pharmaceutical (Tokyo, Japan). All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) in the highest available purity or in HPLC-grade. ODS-Hypersil endcapped (5 μm) was from Merck (Darmstadt, Germany), C_{18} solid-phase extraction columns (3 ml) were from Varian (Frankfurt/Main, Germany). Aqua ad injectabilia in glass containers was from Pfrimmer (Erlangen, Germany).

2.2. Sample preparation and storage

Blood was collected in EDTA-, heparinized or citrated tubes for anticoagulation and centrifuged (3000 *g*, 10 min) immediately after drawing. Plasma samples were stored at −20°C until analysis.

Ocular aqueous humor (50–100 μl) was aspired from the anterior chamber intraoperatively and stored at −20°C until analysis.

2.3. HPLC system

Separation was achieved on a stainless steel column (30 cm×4.6 mm, Bischoff Analysentechnik, Leonberg, Germany) filled with ODS-Hypersil (5 μm, endcapped, Merck). The column was packed according to the "balanced density slurry"-method. Mobile phase was delivered by a Gynkothek 600 HPLC-pump (Gynkothek, Munich, Germany) at a flow-rate of 1.0 ml/min. Samples were applied to the column by means of an automatic sampler (Kontron HPLC autosampler 465, Kontron, Munich, Germany) with a sample volume of 10 μl. Detection at 285 nm was achieved by Kratos Spectroflow 773 photometer with a 8 μl cell (Kratos, Ramsey, NJ, USA). Chromatograms were recorded by a Shimadzu CR6a integrator (Shimadzu, Kyoto, Japan).

The mobile phase was a mixture of 0.01 *M* sodium dihydrogenphosphate monohydrate, acetonitrile (15% by vol.) and N,N-dimethylformamide (6% by vol.) in water with pH adjusted to 3.0 by addition of phosphoric acid (85%). Dissolved gases were removed by 10 min sonication in an ultrasonic bath (Bandelin, Berlin, Germany).

2.4. Preparation of standard solutions

Stock solutions of the four antibiotics and their derivatives (1 mg/ml) were prepared in water. Standard solutions containing the analytes in the concentration range 0.08–20 μg/ml were obtained by dilution with tris-buffer (0.1 *M*, pH 5.0 with hydrochloric acid).

2.5. Analysis of ocular aqueous humor samples

Samples were thawed, centrifuged for 10 min at 3000 *g* and subjected to direct HPLC analysis.

2.6. Solid-phase extraction of plasma samples

C_{18} solid-phase extraction columns were conditioned with 2×3 ml of acetonitrile in a Baker vacuum manifold (10 columns, Baker Chemicals, Deventer, The Netherlands), followed by 3 ml of tris-buffer (0.1 *M*, adjusted with hydrochloric acid to pH 5.0). Two ml of tris-buffer (0.1 *M*, adjusted with hydrochloric acid to pH 5.0) containing 1.25 μg of ciprofloxacine as internal standard were added to 500 μl plasma, and the mixture was applied to the preconditioned extraction columns. After having passed the columns they were washed with 3 ml of tris-buffer (0.1 *M*, adjusted with hydrochloric acid to pH 5.0). For recovery studies blank plasma was spiked with known amounts of the analytes, and processed as described above. Application of vacuum (200 mbar) for 10 min removed traces of humidity prior to elution with subsequent 2×500 μl acetonitrile (40% by vol.) in tris-buffer (0.1 *M*, adjusted with hydrochloric acid to pH 5.0). The eluates were collected in 2.0 ml Eppendorf tubes, and they were then vortexed and subjected to direct analysis.

3. Results

3.1. HPLC

The mobile phase allows baseline separation of the analytes within 15 min (Fig. 2). Retention times of desacetylcefotaxime, cefotaxime, ofloxacine and ciprofloxacine are in the order 3.5, 6.7, 10.2 and 12.0 min, respectively. The retention times of desacetylcefotaxime-lactone and of anti-cefotaxime were determined as 7.8 and 12.8 min, respectively. The standard curves of peak areas against varying concentrations of the analytes in mobile phase were strictly linear for the concentration ranges used (0.08–20 μg/ml):

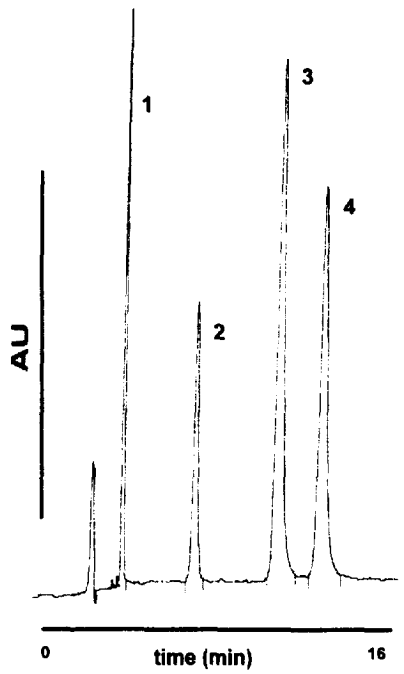

Fig. 2. Chromatogram of the analytes in an aqueous solution (2.5 μg/ml of each compound). (1) Desacetyl-cefotaxime, (2) cefotaxime (3) ofloxacine, (4) ciprofloxacine.

desacetylcefotaxime:	$y=(10\,652\pm28)x+(3373\pm243)$, $(r=0.9999)$;
cefotaxime:	$y=(10\,374\pm22)x+(100\pm195)$, $(r=0.99999)$;
ofloxacine:	$y=(25\,398\pm256)x-(3037\pm2230)$, $(r=0.9998)$;
ciprofloxacine:	$y=(48\,692\pm523)x-(8343\pm4562)$, $(r=0.9997)$.

All determinations were done in 10-fold replication and data for slope and intercept are given with standard deviation. Detection limits for all analytes were at 0.08 μg/ml.

3.2. Solid-phase extraction

Recoveries of the analytes from plasma were determined by spiking a blank plasma with appropriate concentrations of the analytes, followed by the described extraction procedure and HPLC analysis. No interference with endogenous compounds from plasma occurred as shown in Fig. 3. Recovered

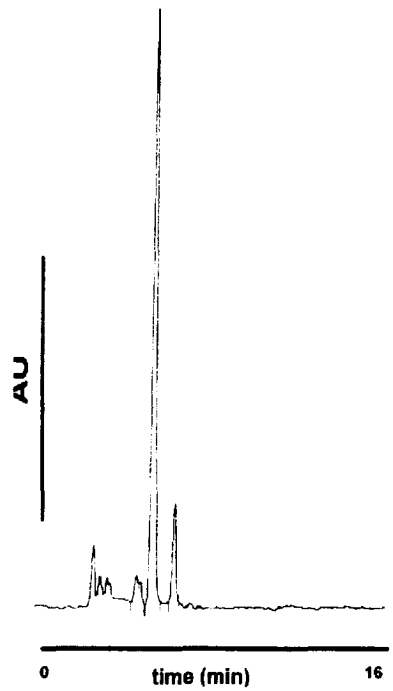

Fig. 3. Chromatogram of a control plasma sample.

concentrations were calculated with respect to the standard curves above for the analytes in mobile phase. The resulting standard curves for spiked plasma samples showed linear characteristics for all analytes (concentrations for all compounds: 0.31–20 μg/ml), given below are the recovery functions (added versus found concentration):

desacetylcefotaxime: $y=(0.80\pm0.005)x-(0.09\pm0.05)$, $(r=0.9999)$;

cefotaxime: $y=(0.95\pm0.003)x-(0.07\pm0.03)$, $(r=0.9999)$;

ofloxacine: $y=(1.00\pm0.003)x-(0.14\pm0.03)$, $(r=0.9999)$;

ciprofloxacine: $y=(1.09\pm0.006)x-(0.03\pm0.05)$, $(r=0.9999)$.

Data for slope and intercept are given with standard deviation. The slope of the different equations indicates the average recoveries of the analytes. At a 100% recovery the added and found concentrations are equal, thus leading to a recovery function with slope 1. The individual recoveries were thus found to be 80% for desacetylcefotaxime, 95% for cefotaxime, 100% for ofloxacine and 109% for ciprofloxacine.

The absolute recovery data with detailed statistics are given in Table 1.

3.3. Internal standardization

Internal standardiation was chosen in order to stabilize the analysis of extracted plasma samples. The standard curve of ciprofloxacine showed linear characteristics for solutions in water as well as for spiked plasma. Therefore this substance was suitable for internal standardization. A constant amount of ciprofloxacine was added to each spiked plasma

Table 1
Absolute and relative recoveries of desacetylcefotaxime, cefotaxime, ofloxacine and ciprofloxacine from spiked plasma after solid-phase extraction and HPLC analysis ($n=10$, mean±S.E.M.)

Substance	Concentration (μg/ml)		Recovery (%)
	Added	Found	
Desacetyl-cefotaxime	0.31	0.70±0.02	224±11.4
	0.63	1.05±0.08	167±2.6
	1.25	1.82±0.02	146±1.3
	2.50	2.99±0.08	120±3.3
	5.00	4.75±0.11	95±2.3
	10.00	8.96±0.21	90±2.1
	20.00	16.67±0.39	83±2.0
Cefotaxime	0.31	0.19±0.01	60±2.1
	0.63	0.50±0.02	80±2.8
	1.25	1.09±0.04	87±3.2
	2.50	2.32±0.08	93±3.3
	5.00	4.76±0.17	95±3.3
	10.00	9.62±0.26	96±2.6
	20.00	18.93±0.61	95±3.0
Ofloxacine	0.31	0.29±0.01	93±4.3
	0.63	0.58±0.02	92±3.2
	1.25	1.15±0.05	92±3.6
	2.50	2.35±0.09	94±3.7
	5.00	4.78±0.13	96±2.1
	10.00	9.63±0.16	96±1.6
	20.00	20.09±0.54	100±2.7
Ciprofloxacine	0.31	0.43±0.02	138±5.1
	0.63	0.71±0.02	112±2.7
	1.25	1.32±0.02	105±1.2
	2.50	2.64±0.03	106±1.4
	5.00	5.35±0.05	107±1.0
	10.00	10.61±0.14	106±1.4
	20.00	21.82±0.38	109±1.9

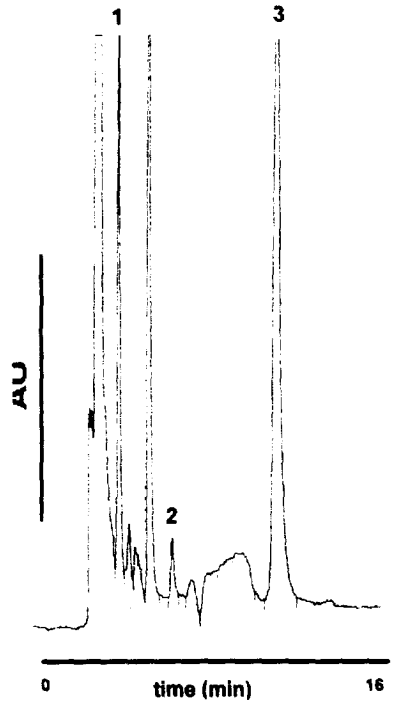

Fig. 4. Chromatogram of an ocular aqueous humor sample from a patient receiving prophylactic antibiotics therapy prior to lens surgery (2 g cefotaxime given intravenously immediately before, and 400 mg ofloxacine given orally 12 h before and, respectively, also immediately before the operation). (1) Desacetylcefotaxime, (2) cefotaxime, (3) ofloxacine.

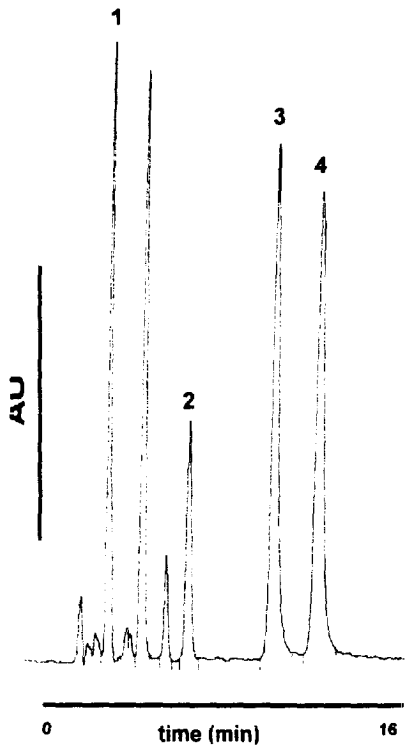

Fig. 5. Chromatogram of a plasma sample from a patient receiving prophylactic antibiotics therapy prior to lens surgery (2 g cefotaxime given intravenously immediately before, and 400 mg ofloxacine given orally 12 h before and, respectively, also immediately before the operation). (1) Desacetylcefotaxime, (2) cefotaxime (3) ofloxacine, (4) ciprofloxacine (1.25 μg added as internal standard).

sample and after solid-phase extraction and HPLC analysis the area quotients of the analytes and ciprofloxacine were taken for quantification. In spiked plasma, these relations were strictly linear for all analytes.

3.4. Application to patients' samples

A patient undergoing prophylactic antibiotic-therapy prior to lens surgery was investigated with this method. A HPLC run of an ocular aqueous humor sample is given in Fig. 4 and a chromatogram of a processed plasma sample is shown in Fig. 5.

4. Discussion

The described HPLC method allows separation and quantification of cefotaxime and its main metabolite desacetylcefotaxime as well as of ofloxacine and ciprofloxacine in one run. The last compound was used as internal standard but may be of interest as an analyte in pharmacokinetic studies. Interference of endogenous compounds in ocular aqueous humor as in extracted plasma samples was excluded. The HPLC method is sensitive and reproducible. Its applicability into clinical samples is demonstrated. The HPLC separation of the antibiotics makes use of the modifying reagent N,N-dimethylformamide for suppression of peak tailing [25] of the fluoroquinolone derivatives which was otherwise (e.g., variation of pH) not achievable. Only the combination of acidic pH for suppression of dissociation of the carboxylic function combined with the modifier for elimination of secondary interactions with the

stationary phase gives acceptable peak shapes. Thus it was possible to develop a HPLC system allowing the sensitive simultaneous determination of both β-lactam- and fluoroquinolone-antibiotics especially valuable in scarce samples as ocular aqueous humor.

Plasma extraction with C_{18}-conjugated solid-phase extraction columns proved to be best suited as compared to other columns based on phenyl-, cyclohexyl- or C_2-conjugated silica. On these columns only low recoveries for the fluoroquinolones were obtained, indicating a weak interaction of these molecules with the modified silica. In contrast, the C_{18}-conjugated silica columns with their long-chained aliphatic, highly lipophilic octadecyl-moiety may have strong interaction with the aliphatic regions of the analytes, as indicated by the recoveries. No interference with endogenous compounds both from ocular aqueous humor and plasma occurred. Recovery of the cefotaximes was easily achieved, only limited by chemical decomposition at pH-values lower than 3 (peak doubling, low recovery). Recovery of the fluoroquinolones was strongly pH-dependent with low recoveries at basic samples' pH due to the acidic structure. The given pH is optimized for both types of antibiotics allowing almost quantitative recovery from plasma samples.

The recovery of desacetylcefotaxime is false positive high in the lower concentration range. This fact was further investigated in order to eliminate the underlying cause. For this reason, chromatograms were recorded with lower flow-rate (0.5 ml/min) than used in the analytical runs. Desacetylcefotaxime has a retention time of 7.6 min under these conditions (Fig. 6a). In Fig. 6c, the chromatogram of an extract of a solution of desacetylcefotaxime in water is shown, revealing the presence of a second compound eluting slightly before desacetylcefotaxime. In the analytical run, this compound is not separated from desacetylcefotaxime but influences the response factor relative to the standard solution, thus leading to the observed high recovery. As in this case all influences of the analytical matrix as plasma or ocular aqeous humor could be excluded, only some degradation of the molecule in the extraction process has to be discussed.

A well known degradation of desacetylcefotaxime is the formation of the lactone [26–28]. In our analytical system, desacetylcefotaxime-lactone is

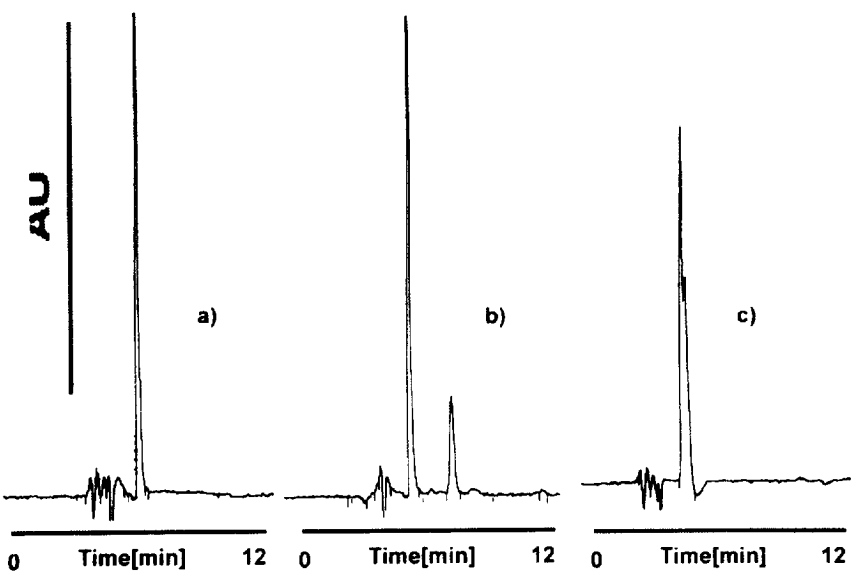

Fig. 6. Chromatograms of desacetylcefotaxime under different conditions: (a) desacetylcefotaxime in water, 2.5 μg/ml, (b) desacetylcefotaxime in water, 2.5 μg/ml after irradiation and (c) desacetylcefotaxime in methanol, 2.5 μg/ml after solid-phase extraction from water under the analytical conditions. Flow-rate, 0.5 ml/min, all other conditions maintained.

eluting at 7.8 min, so it is not relevant. Another well known rearrangement of the cefotaximes is the photodegradation involving *syn*(Z)–*anti*(E) photoisomerisation of the methoxy-imino linkage [29]. As our analytical chromatographic system is able to separate *syn*- and *anti*-cefotaxime (retention times 6.2 resp. 12.8 min), this process may be the reason for the formation of the second peak in the case of desacetylcefotaxime. The anti-derivative of desacetylcefotaxime is not available, therefore we performed photoisomerisation. Irradiation of a solution of desacetylcefotaxime by sunlight for one week indeed provided a second peak eluting at 10.3 min (Fig. 6b) and was accompanied by a distinct yellow discoloration of the solution as described in [29]. This process could also be excluded as a degradation mechanism in our extraction process. Other degradation pathways of cefotaxime and desacetylcefotaxime are described in [27], but the products are unknown. Some catalytic influence of the silica gel may be involved in the degradation mechanism, but could not be avoided in the extraction procedure. So our analytical procedure depends strongly on a carefully performed recovery-control of desacetylcefotaxime in the lower concentration range.

Nevertheless, our proposed analytical method is a useful tool for further comparative investigations of the pharmacokinetics of cefotaxime, ofloxacine and ciprofloxacine. The method is applicable for drug monitoring in patients undergoing prophylactic antibiotics therapy prior to lens surgery.

Acknowledgments

The authors thank Dr. K.G. Schmidt, Department of Ophtalmology, Justus-Liebig-University, Giessen for providing aqueous humor and plasma samples. Approval of the local ethic commission was obtained for performing this study.

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