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Determination of the new fluoroquinolone fleroxacin and its N-demethyl and N-oxide metabolites in plasma and urine by high-performance liquid chromatography with fluorescence detection

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of the new fluoroquinolone fleroxacin and its metabolites in plasma and urine. Plasma samples are deproteinized with acetonitrile, and, after evaporation and reconstitution of the supernatant, samples are analysed on a reversed-phase column. The limit of quantification is 10–20 ng/ml for the parent drug and 10 ng/ml for the metabolites, using a 0.2-ml sample. Urine samples are diluted with the mobile phase. An aliquot is then injected directly onto the column. The limits of quantification are 1 μg/ml for the parent drug and 0.5 μg/ml for the metabolites, using a 0.1-ml sample. The method has been successfully applied to pharmacokinetic studies of human volunteers and patients.

INTRODUCTION

Fleroxacin is a new quinolonecarboxylic acid derivative with potent antibacterial activity against various pathogens, covering gram-positive and gram-negative bacteria [1,2]. Demethylation and N-oxidation have been shown to be the main metabolic pathways in humans [3,4].

Some analytical methods, useful for human pharmacokinetic studies, have been published [5–8]. Three of these are restricted to the determination of the

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parent drug in plasma. The method of Sörgel et al. [8] describes the measurement of the parent drug and its metabolites in plasma; however, certain parameters for method characterization (sensitivity, precision, etc.) are not given.

In order to investigate the pharmacokinetics of the drug and its metabolites, we developed a simple high-performance liquid chromatographic (HPLC) method for the simultaneous measurement of the drug and its metabolites in plasma and urine.

EXPERIMENTAL

Reagents

Fleroxacin (I), its metabolites (II and III) and the internal standard AM 735 (IV) were products from F. Hoffmann-La Roche (Basle, Switzerland) (Fig. 1). Methanol, acetonitrile and KH_2PO_4 (all p.a. grade) were from E. Merck (Darmstadt, F.R.G.). Tetrabutylammonium hydrogensulphate (puriss.) was from Fluka (Neu-Ulm, F.R.G.).

Internal standards. AM 735 (IV) was used as internal standard for the analysis of plasma samples. A stock solution was prepared in 20 m*M* NaOH (0.5 mg/ml). From this stock solution, a working solution (50 ng/ml) for addition to the plasma samples was prepared daily, by dilution in acetonitrile, containing 5% water. Pipemidic acid (V) was used as internal standard for the urine assay. The stock solution was prepared in 10 m*M* HCl. From this, a suitable aqueous working solution (0.1 mg/ml) was prepared daily.

Standard solutions and calibration standards. Stock solutions of compounds I–III were prepared in 20 m*M* NaOH at a concentration of 1 mg/ml. These stock solutions were then diluted further to yield appropriate working solutions for the preparation of the calibration standards.

For the simultaneous determination of I, II and III in plasma, calibration standards were prepared by adding small amounts of the corresponding working solutions to drug-free plasma. The concentration ranges of the calibration standards (containing I, II and III) were 20–5000 ng/ml for I and 10–1000 ng/ml for II and III.

For the exclusive determination of I in plasma, calibration standards were prepared within the range 20–5000 ng/ml.

Urine standards were prepared, containing I, II and III, within the concentration ranges 1–200 μg/ml for I and 0.5–100 μg/ml for II and III.

Apparatus

The HPLC system consisted of the following components: HPLC pumps, Kontron LC 414 T (Kontron, Zürich, Switzerland); autosampler, Gilson 231 (Abimed, Langenfeld, F.R.G.); fluorescence detector, Merck-Hitachi F1000 (Merck, Darmstadt, F.R.G.); computing integrator, SP 4200 with Minifile 4100

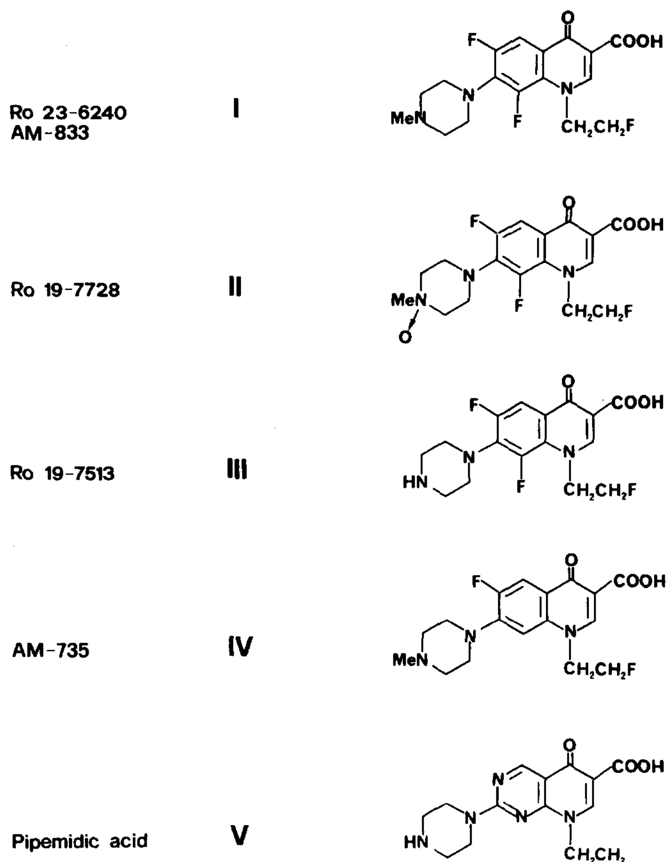

Fig. 1. Structures of fleroxacin (I), its metabolites (II and III) and the internal standards (IV and V).

D (Spectra-Physics, Darmstadt, F.R.G.); HPLC column block heater (Jones, Hengoed, U.K.).

Chromatographic conditions. The column was a 250 mm×4.6 mm I.D. reversed-phase column Toyo Soda, TSK ODS-120T, 5 μm (LKB, Munich F.R.G.). The column temperature was 28°C. The excitation and emission wavelengths of the fluorometric detector were set at 290 and 450 nm, respectively. The mobile phase was 10 m*M* tetrabutylammonium hydrogensulphate–50 m*M* KH_2PO_4 in bidistilled water (solvent A) with methanol (solvent B). The final A–B composition was 72:28 (v/v). The pH of the final eluent was brought to 2.6 with 40% H_3PO_4. The flow-rate was 1.0 ml/min at 10 MPa. The mobile phase was not recycled.

Precolumn. Precolumns were used to project the analytical column and to prevent late-running peaks (occurring in some plasma and urine samples) from

reaching the analytical column. For this, the precolumn was backflushed 1.5 min after sample injection, whereas the flow continued through the analytical column.

Precolumn switching was performed with a Rheodyne Model 7000 air-actuated switching valve. The solenoid valve (Rheodyne Model 7163) was controlled by the external time-events of the Gilson autosampler. Prepacked cartridges (10 mm ×4 mm I.D.) filled with Nucleosil 5-C_{18} were used (Grom, Ammerbuch, F.R.G.). The cartridges were replaced after each daily series (60–80 samples).

Sample preparation

Frozen samples were allowed to thaw at room temperature (darkness) and then homogenized on a vortex mixer.

Plasma. A 200-μl volume of each sample was pipetted into a brown glass tube and 1 ml of acetonitrile containing the internal standard AM 735 was added. After vortex-mixing and centrifuging (10 min, 1000 *g*, 14°C), the supernatant was transferred to another brown glass tube and evaporated under nitrogen (water-bath, 38°C, ca. 20 min). The dry residue was reconstituted in the mobile phase (200 μl for the simultaneous determination of I, II and III, 400 μl for the exclusive determination of I) and transferred to an autosampler vial (glass, capped with teflonized septa), and an aliquot was then injected for analysis (30 out of 200 μl for the simultaneous determination of I, II and III; 20 out of 400 μl for the determination of I).

Urine. An aqueous solution (100 μl) of the internal standard (pipemidic acid) and 5 ml of the mobile phase were added to 100 μl of the urine sample. An aliquot of the resulting mixture was transferred to an autosampler vial (glass, capped with teflonized septa), and 20 μl were injected for analysis.

Calibration and quality control

On each analysis day, six to eight plasma or urine standards, covering the expected concentration range, were extracted as described above and run together with the unknown samples. At the same time, independently prepared quality control samples were extracted.

Calibration was performed by computing a weighted ($1/y^2$) least-squares linear regression ($y=a+bx$; y=peak-height ratio, x=concentration), using a special software package for the SP 4200 computing integrator [9].

RESULTS

Under the chromatographic conditions described, the approximate retention times (min) were 6.0 (V), 7.7 (I), 9.0 (II), 11.5 (III) and 13.0 (IV). Slight differences occurred from column to column.

Internal standard

The internal standard used for the plasma assay could not be used for the urine assay owing to the presence of an endogenous peak in some patients' urines. Pipemidic acid was therefore used for the urine assay.

Linearity

In fluorescence detection, this parameter is strongly detector-dependent and must be tested for each individual detector. Under the conditions described, and following the above procedures, the standard curves were linear (r^2 better than 0.99) over the concentration ranges described above. Samples with concentrations above the linear range were diluted further with drug-free plasma or urine.

Limits of quantification

Plasma assay. The sensitivity of the fluorescence detector with respect to baseline noise would allow a quantification limit of ca. 1 ng/ml for compound I. However, there has previously been no need to measure concentrations of I below 10–20 ng/ml. Therefore, the lowest calibration standards (used for the determination of I) were 10 or 20 ng/ml (R.S.D. = 7.5%).

The fluorescence sensitivity for compounds II and III is less than that for I (by a factor of 2.5). The practical limit of quantification was 10 ng/ml (R.S.D. = 9%) for II and III.

Urine assay. The lowest calibration standards were 1 μg/ml for I (R.S.D. = 4%) and 0.5 μg/ml for II and III (R.S.D. = 5%). As for the plasma assay the lowest calibration standards were not identical with the possible quantification limits.

Precision

Inter-assay (day-to-day) coefficients of variation (C.V.) were calculated from the quality control samples processed with the daily unknown samples. Mean C.V. values for the plasma assay were 5.5% for compound I (range 20–5000 ng/ml) and compounds II and III (range 10–1000 ng/ml) (Table I). Mean C.V. values for the urine assay were 4% for compound I (range 1–200 μg/ml) and compounds II and III (range 0.5–100 μg/ml) (Table II).

Selectivity

The use of fluorescence as opposed to UV detection nearly always increases the selectivity of a method. Other quinolones, such as pefloxacin, ciprofloxacin and norfloxacin, did not interfere with the analysis for fleroxacin in plasma or urine. In any case, it is extremely unlikely that two or more quinolones would be co-administered.

TABLE I

PRECISION (DAY-TO-DAY) AND ACCURACY OF THE PLASMA ASSAY

Obtained from quality control samples.

Compound	Concentration (ng/ml)		Deviation added – found (%)	C.V. (%)	n
	Added	Found			
I	20	19.3	−3.5	8.5	8
III	10	10.3	+5.0	8.0	10
II	10	10.3	+3.0	7.6	4
I	200	192	−4.0	5.7	21
III	100	103	+3.0	5.6	20
II	100	102	+2.0	6.1	19
I	5000	4780	−4.4	4.1	13
III	1000	1030	+3.0	5.2	13
II	1000	1020	+2.0	6.3	13

TABLE II

PRECISION (DAY-TO-DAY) AND ACCURACY OF THE URINE ASSAY

Obtained from quality control samples.

Compound	Concentration (μg/ml)		Deviation added – found (%)	C.V.	n
	Added	Found			
I	1	0.94	−6.0	4.4	7
III	0.5	0.48	−4.0	5.0	7
II	0.5	0.50	0.0	5.2	7
I	20	19.7	−1.5	3.9	13
III	10	10.0	0.0	3.7	13
II	10	10.2	+2.0	3.8	13
I	200	197	−1.5	4.0	13
III	100	100	0.0	3.5	13
II	100	104	+4.0	3.6	13

Stability

Fleroxacin and its metabolites, like other quinolones, are light-sensitive [6]. Each sample manipulation step (thawing, pipetting, etc.), therefore, must take place rapidly in a darkened room. We recommend the use of brown glass vessels for the sample preparation steps.

Compounds I, II and III were found to be stable at −20°C in the dark for

four months (plasma) and six months (urine) [6]. Following protein precipitation, the compounds (including the internal standard AM 735) were stable in the eluent (darkened room, room temperature) for at least 24 h.

Extraction efficiency

Following the whole sample preparation procedure, the recoveries from plasma were 98% for I (range 50–5000 ng/ml), 77% for III and 49% for II (range 50–500 ng/ml).

DISCUSSION

Chromatography

Compounds of the quinolone type may theoretically be chromatographed on reversed-phase systems under the following conditions: (i) as protonated quinolones under acidic conditions, thereby suppressing ionization of the carboxylic group; (ii) by ion-pairing the basic part of the molecule (e.g. with heptanesulphonic acid [10] or by ion-pairing the acidic part of the molecule (e.g. with tetrabutylammonium salts).

The use of quaternary ammonium salts as 'ion-pairing' compounds has been described [6,11,12,13]. We are of the opinion that, under these conditions (acidic eluent), quaternary ammonium salts act more as 'anti-tailing' agents (competing for the available adsorption sites on the stationary phase) than as ion-pairing compounds. For instance, the use of anti-tailing agents, such as triethylamine, in the eluent has been described for the determination of quinolones by reversed-phase chromatography [5,14,15]. Most authors who have

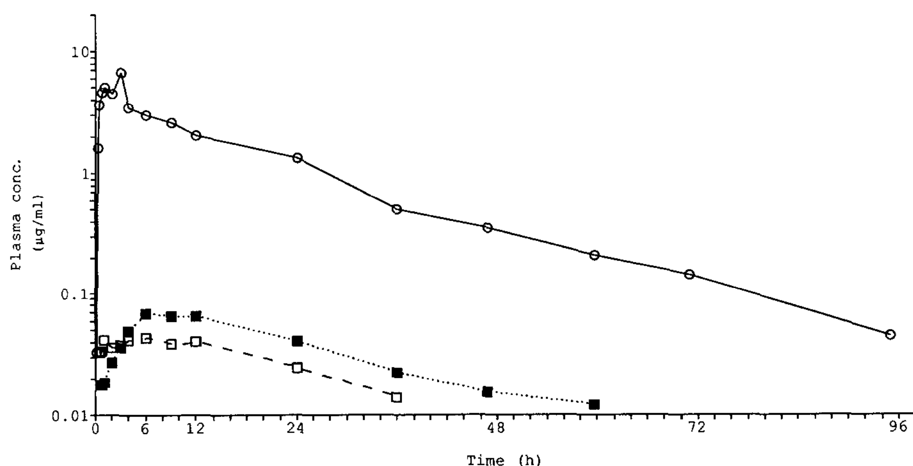

Fig. 2. Plasma concentration of fleroxacin (○) and its metabolites N-demethylfleroxacin (□) and fleroxacin N-oxide (■) following oral (400 mg) application.

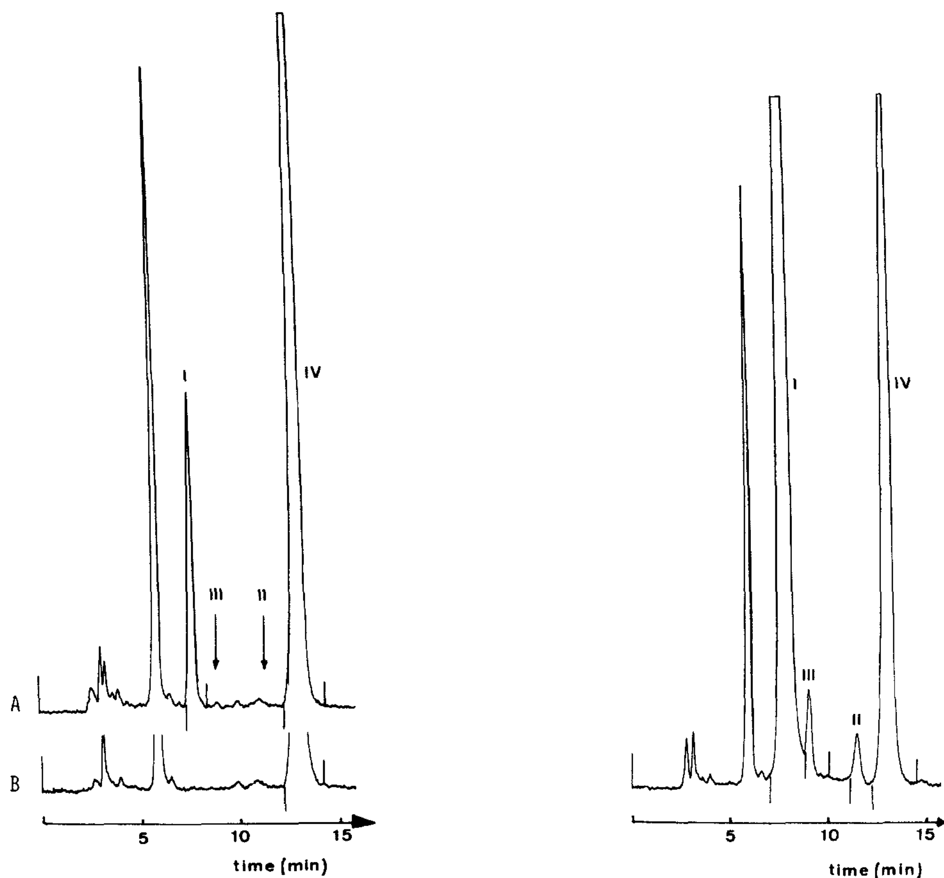

Fig. 3. (A) Chromatogram of a plasma sample 96 h after a 400-mg oral dose of fleroxacin to a healthy subject; concentrations: I, 38 ng/ml, III and II, below 10 ng/ml; IV = internal standard. Injected volume: 30 out of 200 μl (see *Sample preparation*). (B) Chromatogram of the corresponding pre-dose sample with added internal standard.

Fig. 4. Chromatogram of a plasma sample 24 h after a 400-mg oral dose to a healthy subject. Concentrations: I, 1130 ng/ml; III, 34 ng/ml; II, 24 ng/ml; IV = internal standard.

described the use of quaternary ammonium salts or basic modifiers in the mobile phase also used anionic compounds, such as phosphate or acetate ions, which might also form ion-pairs with a quinolone in its protonated form.

Because their molecules contain both acidic and basic centres, quinolones are critical compounds in reversed-phase chromatography. Dell et al. [6] found that the best peak shapes for fleroxacin were obtained with the Toyo Soda material. We used almost identical chromatographic conditions. A slight improvement in peak shape was obtained by increasing the salt concentration (addition of KH_2PO_4) in the eluent. Optimal peak shapes were obtained only after a new column had been equilibrated with the mobile phase for at least 24

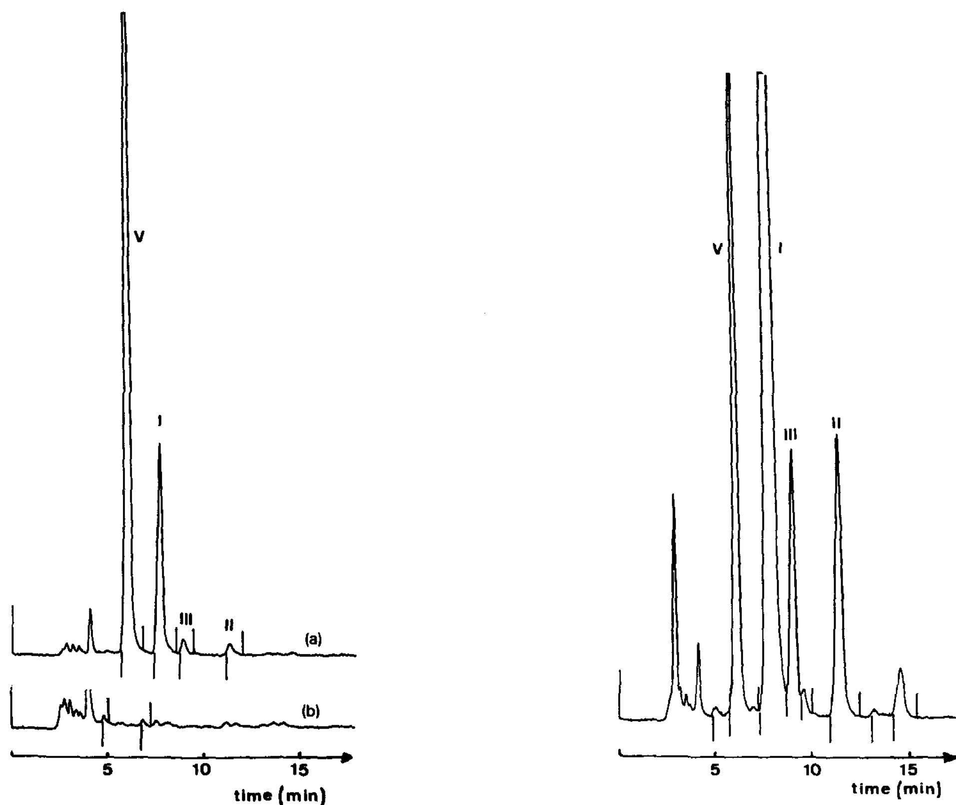

Fig. 5. (a) Chromatogram of a 48–72 h urine sample following a 400-mg oral dose of fleroxacin to a healthy subject; concentrations: I, 7.3 μg/ml; III, 1.14 μg/ml; II, 0.65 μg/ml; V = internal standard. (b) Chromatogram of a corresponding pre-dose sample (not containing internal standard).

Fig. 6. Chromatogram of a 0–6 h urine sample following a 400-mg oral dose of fleroxacin to a healthy subject. Concentrations: I, 134 μg/ml; III, 9.7 μg/ml; II, 10.6 μg/ml; V = internal standard.

h. Although not absolutely necessary, the use of a column heater (28°C) had the advantage of shortening the retention times and maintaining stable conditions.

Quantification of the N-demethyl metabolite

Especially in the case of plasma samples, concentrations of fleroxacin are much greater than those of the metabolites. Under these conditions, the peaks for fleroxacin (I) and for the N-demethyl metabolite (III) are not separated to the baseline (see Fig. 4) and a skimming function should be used to measure the correct peak height of compound III. In the analysis of artificial mixtures of fleroxacin and its N-demethyl metabolite (100:1 and 200:1), the use of this integration procedure was shown to yield correct results.

Sample preparation

The reported sample preparation procedures for quinolones vary from very extensive (double extractions, followed by back extraction) [10] to very simple (protein precipitation and injecting the supernatant) [11,15,16]. The extraction procedure described for compound I by Dell et al. [6] led to insufficient extraction of the metabolites II and III.

We first tried 3% aqueous trichloroacetic acid as protein-precipitating agent and injected an aliquot of the supernatant, mixed with an equal volume of the mobile phase. However, unstable chromatographic conditions sometimes occurred, especially when a relatively large amount of the acidic medium had to be injected for the determination of the metabolites. Subsequently, protein precipitation with acetonitrile was used, followed by evaporation of the supernatant, and taking up the residue in the mobile phase. The procedure is simple and allows 60–80 samples (calibrations included) to be analysed per day.

Application

The average lifetime of an analytical column was ca. 3000 injections (use of precolumns recommended), whereby the column flow direction was reversed after a certain time (because of peak broadening). The inlet frits were cleaned when the column back-pressure increased to above 20 MPa.

The method has been applied to the analysis of samples from human pharmacokinetic studies, including patients and volunteers. Plasma profiles from a representative subject (Fig. 2) and chromatograms of the plasma and urine assay (Figs. 3–6) are shown.

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REFERENCES

1 K. Hirai, H.A. Hoyama, M. Hosaka, Y. Oomori, Y. Niwata, S. Suzue and T. Irikura, Antimicrob. Agents Chemother., 29 (1986) 1059–1066.
2 N. Manek, J.M. Andrews and R. Wise, Antimicrob. Agents Chemother., 30 (1986) 330–332.
3 M. Nakashima, M. Kanamaru, T. Uematsu, A. Takiguchi, A. Mizuno and J. Itaya, J. Antimicrob. Chemother., 22 (1988) 133–144.
4 E. Weidekamm, K. Stöckel and D. Dell, Drug Exp. Clin. Res., 13 (1987) 85–90.
5 W.M. Awni, J.A. Maloney and K.L. Heim-Duthey, Clin. Chem., 34 (1988) 2330–2332.
6 D. Dell, C. Partos and R. Portmann, J. Liq. Chromatogr., 11 (1988) 1299–1312.
7 R. Wise, B. Kirkpatrick, J. Ashby and D.J. Griggs, Antimicrob. Agents Chemother., 31 (1987) 161–163.
8 F. Sörgel, R. Seelmann, K. Naber, R. Metz and P. Muth, J. Antimicrob. Chemother., 22 (1988) 169–178.

9 U. Timm, D. Dell and D.A. Ord, Intell. Instrum. Comput., 3 (1985) 29–35.
10 L.T. Pauliukonis, D.G. Musson and W.F. Bayne, J. Pharm. Sci., 73 (1984) 99–102.
11 B. Joos, B. Ledergerber, M. Flepp, J.D. Bettex, R. Lüthy and W. Siegenthaler, Antimicrob. Agents Chemother., 27 (1985) 353–356.
12 F. Jehl, C. Gallion and J. Debs, J. Chromatogr., 339 (1985) 347–357.
13 A.J.N. Groeneveld and J.R.B.J. Brouwers, Pharm. Weekbl. Sci. Ed., 8 (1986) 79–84.
14 G. Montay and J.P. Tassel, J. Chromatogr., 339 (1985) 214–218.
15 T.B. Vree, J. Chromatogr., 343 (1985) 449–454.
16 A. Weber, D. Chaffin, A. Smith and K.E. Opheim, Antimicrob. Agents Chemother., 27 (1985) 531–534.