Journal of Chromatography, 416 (1987) 321–330
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3583

SENSITIVE AND SELECTIVE DETERMINATION OF PICOGRAM AMOUNTS OF CIPROFLOXACIN AND ITS METABOLITES IN BIOLOGICAL SAMPLES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PHOTOTHERMAL POST-COLUMN DERIVATIZATION

H. SCHOLL*, K. SCHMIDT and B. WEBER

Bayer AG, EP-AQ-Forschungsanalytik, Postfach 10 17 09, D-5600 Wuppertal 1 (F.R.G.)

(First received September 22nd, 1986; revised manuscript received January 14th, 1987)

SUMMARY

An analytical method for the detection and quantification of ciprofloxacin and its known metabolites M1, M2, M3 and M4 in urine, serum/plasma, bile, faeces and tissue is described. For the liquid matrices the only sample preparation that is required is dilution. The assay consists of reversed-phase high-performance liquid chromatography and fluorescence detection. For the metabolites M2, M3 and M4 an additional post-column derivatization by successive thermolysis and photolysis is needed. A suitable simple post-column reactor has been constructed. Detection limits for all compounds are between 0.2 and 2.2 ng/ml (absolute detection limits 2–22 pg). Compared with fluorescence detection alone, the derivatization increases the selectivity of detection significantly. The linearity, precision and accuracy of the method were determined.

INTRODUCTION

The known high-performance liquid chromatographic (HPLC) methods of ciprofloxacin analysis are almost entirely restricted to quantification of ciprofloxacin [1–7]. Only two existing papers are concerned additionally with the known metabolites (three in each case, Fig. 1) [8, 9]. No HPLC method has yet been published by which all four known metabolites can be determined with the same sensitivity and selectivity as ciprofloxacin. This is due not to a chromatography problem but to a detection problem: although fluorescence detection is very sensitive for ciprofloxacin and M1 it is much less sensitive for M3 and M4 (Table I). The fluorescence of M2 is so weak, 2800 times weaker than that of M1, that UV detection, which for the other metabolites is less selective and less sensitive, is preferable to fluorescence detection.

0378-4347/87/$03.50

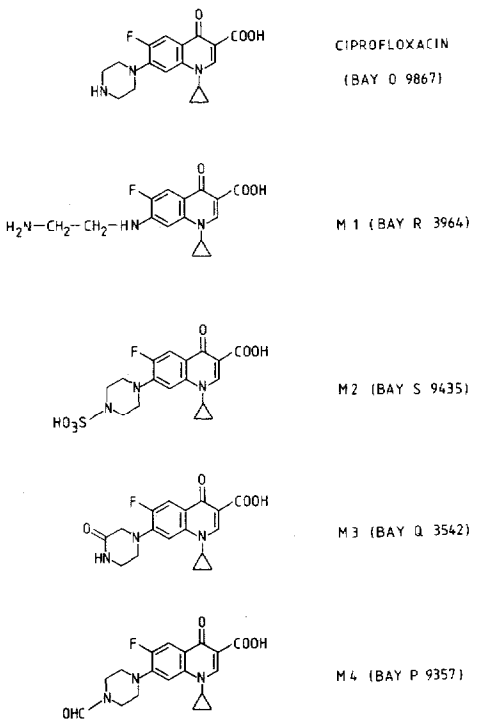

Fig. 1. Ciprofloxacin and its metabolites.

To seek a solution to this problem we employed post-column derivatization in an attempt to convert metabolites M2, M3 and M4 into secondary products with fluorescence comparable with that of ciprofloxacin. In order to study the influence of the widest possible range of reaction conditions we designed a reaction system that permits both photolysis and thermolysis. The structural requirements of such a system are evident from the work of Scholten et al. [10], who demonstrated the feasibility of UV irradiation of eluents flowing through PTFE capillaries. Other designs for various types of photolysis reactor have been published by Twitchett et al. [11], Uihlein and Schwab [12] and Verbeke and Vanhee [13]. Verbeke and Vanhee described an additional hydrolysis step (for a comprehensive survey of post-column derivatization in chromatography see refs. 14 and 15).

EXPERIMENTAL

Reagents

All reagents were of analytical grade and were obtained from Merck (Darmstadt, F.R.G.). Tetrabutylammonium bisulphate was obtained from Sigma (St. Louis, MO, U.S.A.). The standard substances used for quantitative analysis were Bay R 3964·HCl, assay 96% (M1); Bay O 9867·HCl·H_2O, assay 98.3%; Bay S 9435·NH_3, assay 90.3% (M2); Bay Q 3542, assay 99% (M3) and Bay P 9357, assay 99% (M4), and were obtained from Bayer (Leverkusen, F.R.G.).

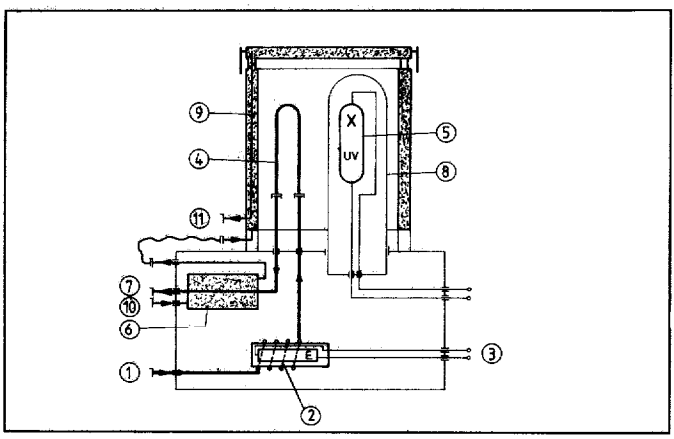

Fig. 2. Principle of post-column derivatization. For description of numbers see text.

Chromatography

An SP 8100 high-performance liquid chromatograph with an SP 8110 autosampler and 4200 integrator (Spectra-Physics, Palo Alto, CA, U.S.A.) with an LS 4 fluorescence detector (Perkin-Elmer, Überlingen, F.R.G.) was used. The stationary phase was Nucleosil 120-5 C_{18}, 5 μm, Batch No. 5030 (Macherey Nagel, Düren, F.R.G.) in a stainless-steel column (250 mm×4 mm I.D.). The mobile phase was a mixture of 40 g of tetrabutylammonium bisulphate per litre of doubly distilled water and acetonitrile, the latter being in a proportion of either 7–10 vol. % or 25–30 vol. %. The flow-rate was 2 ml/min and the oven temperature 40°C. Detection was at an excitation wavelength of 278 nm (slit width 10 nm) and an emission wavelength of 445 nm (slit width 20 nm). The injection volume was 10 μl. The UV detection limits were determined with an SP 8440 detector (Spectra-Physics).

An alternative solvent system employed for post-column derivatization consisted of methanol and phosphate buffer. The buffer contained 9.07 g of potassium dihydrogen phosphate per litre of water, and was adjusted to pH 3 with phosphoric acid; the proportion of methanol in the mixture was 20 vol. %.

Post-column derivatization

For the post-column derivatization a special reactor was designed. Its principle of operation is shown in Fig. 2. On emerging from the column (1) the eluate passes through a stainless-steel capillary (250 mm × 0.2 mm I.D.) (2) in which it is heated by an adjustable heating element (3), and then into a PTFE capillary (200 mm × 0.5 mm I.D. × 1 mm O.D.) (4) in which it is irradiated by a high-pressure mercury lamp HPK 125 WL (Philips, Hamburg, F.R.G.) (5), through a cooler (6) and hence to the fluorescence detector (7). The distance between the lamp and the PTFE capillary is 30 mm, the lamp is housed inside a protective quartz envelope (8). The lamp and PTFE capillary are enclosed by a cooled double-walled nickel-plated brass cylinder of 55 mm I.D. (9). Cooling is by means of tap water at 5–15°C which enters at 10 and emerges at 11. The lamp yields a

continuous spectrum with maxima at 270 and 313 nm and additional emission lines at 254/8, 313 and 366 nm. The maximum of UV absorption of the metabolites is at 278 nm.

Sample preparation

All samples were filtered through a PTFE membrane filter (pore size 0.45 μm) before chromatography. Liquid matrices were stirred for 10 s with a Vibrofix F4 (Jahnke and Kunkel, Staufen, F.R.G.) and immediately diluted to the concentration range of the calibration solutions with 0.033 *M* phosphoric acid. Bile samples were additionally homogenized with a Labsonic 15-10 ultrasonic homogenizer (Braun, Melsungen, F.R.G.) for three 10-s intervals before dilution.

Faeces samples were mixed with two to four times their volume of water and homogenized with an Ultra-Turrax (Jahnke and Kunkel). Aliquots of ca. 100 g were accurately weighed into 1000-ml brown glass bottles, treated with 500 ml of a mixture of dichloromethane–propan-2-ol–0.3 *M* phosphoric acid (1:5:4, v/v/v) and extracted on a shaking machine for 1.5 h at room temperature. A 4-ml aliquot was then centrifuged, and the supernatant was diluted with 0.033 *M* phosphoric acid and chromatographed. The results were calculated on the assumption that the 100-g aliquots had a density of 1 g/ml.

For the extraction of tissue, 200 mg of tissue and 5 ml of the extraction solution were homogenized with an Ultra-Turrax for 2 min. The homogenate was centrifuged and the supernatant was filtered and chromatographed. The extraction solution consisted of 12.5 ml of concentrated perchloric acid and 2 g of concentrated phosphoric acid, which were diluted to a final volume of 1000 ml with water–methanol (1:1, v/v).

Quantification

The external-standard method was employed. All the standard solutions were prepared by diluting a stock solution with 0.033 *M* phosphoric acid. The stock solutions were prepared by accurately weighing ca. 5 mg of the appropriate standard substance, dissolving it in 5 g of concentrated phosphoric acid, diluting with 10 ml of acetonitrile and making up to 50 ml with water.

Sixty-four biological samples and sixteen standard samples were measured in each run. The sixteen standard samples consisted of four sets of four standard solutions of different concentration, which were spaced at equal distances between the biological samples in the autosampler and chromatographed. The calibration line was constructed from the means of the four readings obtained for each standard concentration.

To determine the detection limits (Tables I and II) the stock solutions of the five substances were diluted with 0.033 *M* phosphoric acid until the signal-to-noise ratio of the chromatogram was 3:1. The corresponding concentrations were taken as detection limits and, for an injection volume of 10 μl, refer to the concentrations used (in ng/ml) or to the absolute amounts injected (in pg).

All the standard solutions in 0.033 *M* phosphoric acid are stable for 18 h in the autosampler, seven days in a refrigerator at ca. 4 °C and thirty days deep-frozen at −20 °C. Stability limits were not determined.

TABLE I

CIPROFLOXACIN AND METABOLITES: COMPARISON OF DETECTION LIMITS FOR UV AND FLUORESCENCE DETECTION

For HPLC conditions, see Experimental.

Substance	Detection limit			
	UV		Fluorescence	
	ng/ml	pg	ng/ml	pg
Ciprofloxacin	140	1400	1.8	18
M1	85	850	0.10	1
M2	74	740	280	2800
M3	29	290	7.4	74
M4	45	450	20	200

Linearity, precision and accuracy of the determination by post-column derivatization

To determine the linearity of the calibration function the stock solutions of M2, M3 and M4 were diluted to seven to ten different concentrations with 0.033 *M* phosphoric acid, which were each measured five times.

The accuracy of the method was determined by recovery measurements; known amounts of the standard substances were added in solution to the blank matrix, worked up and analysed. The solutions used for this purpose were prepared in the same way as the standard solutions and were added in volumes smaller than 1% of the volume of the blank matrix. The amounts used were chosen so as to give, for all matrices except faeces, at least one group with high concentrations and one group with low concentrations. For faeces only a medium concentration was measured. At least three independent samples were prepared and analysed for each concentration of each individual substance. The ranges of the recovery concentrations were: high, 100 μg/ml; medium, 100 ng/ml; low, 10 ng/ml.

TABLE II

CIPROFLOXACIN AND METABOLITES: INFLUENCE OF POST-COLUMN DERIVATIZATION (PCD) ON DETECTION LIMITS

Substance	Detection limit			
	Without PCD		With PCD	
	ng/ml	pg	ng/ml	pg
Ciprofloxacin	1.8	18	1.1	11
M1	0.10	1	0.23	2.3
M2	280	2800	2.2	22
M3	7.4	74	0.33	3.3
M4	20	200	0.64	6.4

RESULTS AND DISCUSSION

Post-column derivatization

For the detection of M2, M3 and M4 the new post-column derivatization procedure is a distinct improvement on fluorimetry. The detection limit for M2, 2.2 ng/ml (22 pg absolute, see Table II) instead of the previous 280 ng/ml, is lower by a factor of 130. For M1 and ciprofloxacin, on the other hand, the difference is only small, but this is of no consequence since both substances fluoresce strongly anyway without post-column derivatization. Hence the detection of metabolites from ballast-rich matrices can be much more selective. Whereas, without post-column derivatization the detection sensitivity is insufficient to determine the metabolites against the background of the matrix components, the additional procedure converts the metabolites into intensely fluorescing secondary products that are easy to distinguish from the matrix components and to quantify.

Optimization of the reaction conditions for post-column derivatization of ciprofloxacin metabolites revealed that M3 and M4 essentially react to changes in photolysis time whereas M2 additionally responds stronger to an increase in thermolysis temperature. The optimum conditions for the conversion of all three metabolites in the apparatus employed in the present study are (1) a PTFE capillary length of 200 mm for the photolysis and (2) heating the eluent just below the boiling point for thermolysis. Under these conditions the reaction time is 0.6 s for photolysis and 2 s for thermolysis. An absolute amount of 2.3–22 pg is detectable for all metabolites.

The lifetime of the PTFE capillary is more than twenty weeks of operation and seems to be not significantly affected by the irradiation or the eluent.

For ciprofloxacin metabolites the improvement in fluorescence detection obtained with post-column derivatization is not restricted to the solvent system consisting of acetonitrile and tetrabutylammonium bisulphate in water. Comparative measurements in the HPLC system described by Krol et al. [9], and moreover in a solvent system consisting of methanol–phosphate buffer (pH 3), led to a comparable increase in fluorescence yield without optimization by additional post-column derivatization. Use of post-column derivatization should therefore prove beneficial in the determination of ciprofloxacin metabolites in all the reported HPLC systems for ciprofloxacin that operate with acid solvents [1–9].

The combination of photolysis and thermolysis permits variation of the thermolysis conditions (by changing the temperature) and photolysis (by changing the dimensions of the PTFE capillary or the flow-rate) and is hence possibly applicable to other substances besides ciprofloxacin and the other gyrase inhibitors with quinolone carboxylic acid structures. The range of experimental conditions could be extended further by using different UV lamps (e.g. "cold" lamps [12]) or extremely long "knitted" PTFE capillaries [16]. Photolysis and thermolysis may be used in the reverse sequence.

The post-column derivatization is linear in the tested ranges of 2.4–1925 ng/ml for M2, 2.6–258 ng/ml for M3 and 1.3–1040 ng/ml for M4.

Averaged coefficients of variation of n independent determinations were 1.7%

TABLE III

CIPROFLOXACIN AND METABOLITES: RECOVERIES AND RANGES FOR URINE, SERUM, FAECES, BILE AND TISSUE

Values are overall means of three independent individual determinations for each of the concentrations of 100 μg/ml, 100 ng/ml and 10 ng/ml; for faeces 100 ng/ml.

Matrix	Recovery (%)				
	Ciprofloxacin	M1	M2	M3	M4
Urine	98.9 98.0-99.5	92.5 85.8-97.1	99.8 93.5-102.5	100.5 93.8-107.9	95.3 92.2-97.5
Serum	99.5 95.3-103.8	106.9 97.1-116.7	104.9 104.4-105.8	98.5 94.3-102.3	88.3 83.7-92.8
Bile	96.3 91.7-99.2	98.3 91.2-102.1	96.0 95.1-98.0	97.8 94.1-99.9	95.1 89.7-96.5
Tissue	95.8 89.3-101.7	92.2 84.0-98.3	97.4 93.2-101.4	87.2 78.4-93.7	86.0 71.3-100.8
Faeces	89.5	87.5	93.8	94.9	89.7

for M2 ($n=50$), 2.3% for M3 ($n=35$) and 2.7% for M4 ($n=40$), without significant differences between high and low concentrations of the linear range.

No isolation or clarification of the structure of the strongly fluorescing reaction products formed in the post-column derivatization has yet been attempted.

Chromatography

The method of Gau et al. [8] was modified in order to allow all known metabolites to be determined in addition to ciprofloxacin itself. Nucleosil was used as the stationary phase instead of ODS II. Tetrabutylammonium bisulphate was used instead of tetrabutylammonium hydroxide so that the aqueous component of the solvent system had a constant acid pH of ca. 2 without additional acidification with phosphoric acid.

Chromatography was performed isocratically with two different solvent systems comprising 4% aqueous tetrabutylammonium bisulphate solution with 7–10% acetonitrile for M1 and ciprofloxacin and 25–30% acetonitrile for M2, M3 and M4. The approximate retention times were 3.5 min for M1, 4.5 min for ciprofloxacin, 6 min for M2, 2.5 min for M3 and 3.5 min for M4; thus the entire chromatography was complete in less than 15 min (Fig. 3).

This procedure is quicker and more reliable than the separation of all five substances in only one run, which requires gradient elution and reconditioning of the column roughly every 45 min. The differences in polarity between the metabolites are too large for a single isocratic solvent system.

The longer lifetime of the columns and the lower risk of protein precipitates associated with the use of tetrabutylammonium salts in the solvent system [8] were confirmed for tetrabutylammonium bisulphate. Between 300 and 1500 sep-

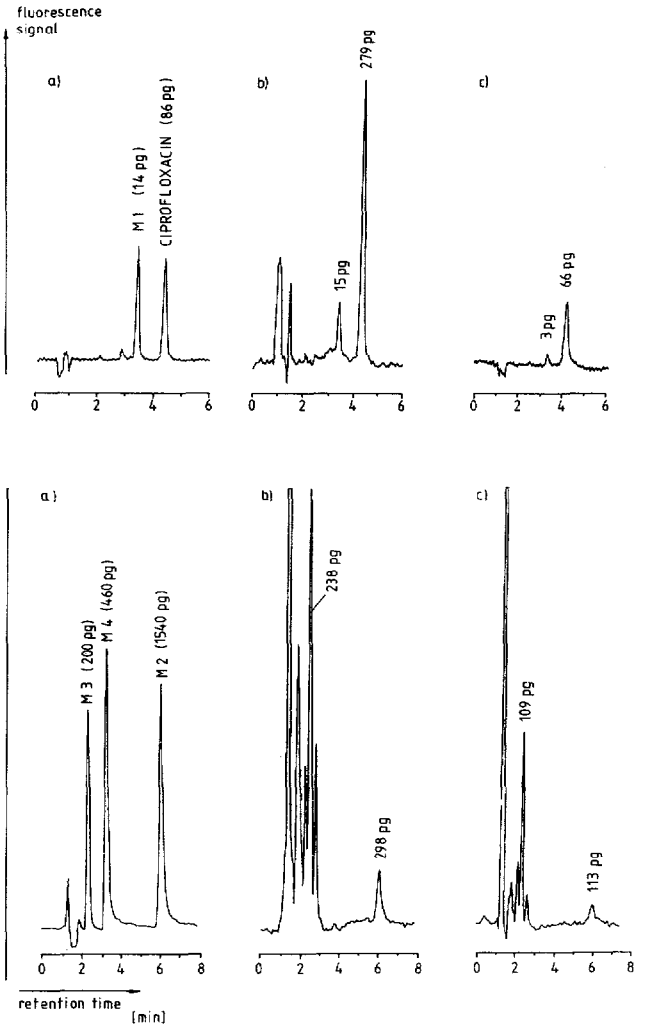

Fig. 3. Chromatography of ciprofloxacin and its metabolites with post-column derivatization: injection of (a) standard solutions, (b) serum after dilution by a factor of 3.33 (top) or 5 (bottom) and (c) urine after dilution by a factor of 2500 (top) or 2000 (bottom).

arations, depending on dilution and matrix, can be performed without loss of separation capacity.

The separation and quantification of metabolites were extended from urine, serum and plasma to bile, faeces and various tissues. As in the work of Gau et al. [8], sample preparation was largely dispensed with. Liquid matrices were chromatographed directly after dilution with 0.033 *M* phosphoric acid. Only solid matrices were extracted before chromatography, enhancement of the lipophilicity of the extractant by addition of methylene chloride being necessary for faeces. Similarly the addition of perchloric acid as a protein-precipitating reagent in the extraction of tissues gives a higher extraction yield, which is more easily reproducible.

In the recovery experiments more than 90% of the administered amount was recovered from the principal matrices as ciprofloxacin or its metabolites (Table III).

As far as possible the selectivity of the chromatography system used for these experiments was ensured by working up and measuring all the blank samples that became available in the course of our routine analysis for each matrix (currently a total of more than 400 individual blank samples). No appreciable interferences of endogenous substances with ciprofloxacin or its metabolites were detected. Concentrations of M4 are so far generally below the detection limit (see Fig. 3b and c, lower panel).

CONCLUSIONS

A rapid chromatographic method has been developed that is suitable for the separation and quantitative determination of ciprofloxacin and its known metabolites from various matrices and requires minimal sample preparation. The post-column derivatization system that was developed to improve the detection is not restricted to ciprofloxacin. The example of the ciprofloxacin metabolites has shown that it provides highly sensitive, selective and precise detection of substances in the parts per 10^9 range, which otherwise fluoresce weakly or not at all.

ACKNOWLEDGEMENTS

We are grateful to Herr H. Horn and his colleagues for building the post-column derivatization apparatus, Herr R. Müller for carrying out the measurements and Herr Dipl.-Ing. H.-J. Hohmann for providing Fig. 2.

REFERENCES

1 F. Jehl, C. Gallion, J. Debs, J.M. Brogard, H. Monteil and R. Minck, J. Chromatogr., 339 (1985) 347.
2 D.E. Nix, J.M. de Vito and J.J. Schentag, Clin. Chem., 31 (1985) 684.
3 C.E. Fasching and L.R. Peterson, J. Liq. Chromatogr., 8 (1985) 552.
4 G. Höffken, H. Lode, C. Prinzing, K. Borner and P. Koeppe, Antimicrob. Agents Chemother., 27 (1985) 375.
5 B. Joos, B. Ledergerber, M. Flepp, J.-D. Bettex, R. Lüthy and W. Siegenthaler, Antimicrob. Agents Chemother., 27 (1985) 353.
6 A.Weber, D. Chaffin, A. Smith and K.E. Opheim, Antimicrob. Agents Chemother,. 27 (1985) 531.
7 A.J.N. Groeneveld and J.R.B.J. Brouwers, Pharm. Weekbl. (Sci.), 8 (1986) 79.
8 W. Gau, H.-J. Ploschke, K. Schmidt and B. Weber, J. Liq. Chromatogr., 8 (1985) 485.
9 G.J. Krol, A.J. Noe and D. Beerman, J. Liq. Chromatogr., 9 (1986) 2897.
10 A.H.M.T. Scholten, P.L.M. Welling, U.A.Th. Brinkmann and R.W. Frei, J. Chromatogr., 199 (1980) 239.
11 P.J. Twitchett, P.L. Williams and AC. Moffat, J. Chromatogr, 149 (1978) 683.
12 M. Uihlein and E. Schwab, Chromatographia, 15 (1982) 140.
13 R. Verbeke and P. Vanhee, J. Chromatogr., 265 (1983) 239.
14 I.S. Krull, C.M. Selavka, C. Duda and W. Jacobs, J. Liq. Chromatogr., 8 (1985) 2845.

15 I.S. Krull, Post-Column Reaction Detectors in HPLC, Marcel Dekker, New York, in press
16 V.-D. Neue, Dissertation, Saarbrücken, 1976.