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Determination of BAY y 3118, a novel 4-quinolone, in biological fluids using high-performance liquid chromatography and photothermal post-column derivatization

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method that allows the sensitive and selective quantification of a novel 4-quinolone (BAY y 3118, I) in biological fluids is described. After sample dilution with 0.05 *M* phosphoric acid (plasma) or 0.1 *M* phosphate buffer pH 7.5 (urine), samples can be directly injected into the HPLC system. Prior to fluorescence detection, I is decomposed to fluorescence compound(s) by post-column derivatization utilizing either photolysis (Beam Boost reaction unit) or a combination of thermolysis and photolysis (laboratory-made post-column reactor). Compared with fluorescence detection alone, derivatization increases the signal intensity (about 80-fold) and the selectivity of the detection significantly. Concentrations down to 0.01 mg/l could be quantified in biological fluids. Only thermolysis was not able to decompose I to fluorescence products. Investigations on the stability of I in plasma and urine demonstrate good stability under the different conditions tested. The method was applied to human plasma and urine samples from a subject after a single oral dose of 100 mg of I.

INTRODUCTION

BAY y 3118, 1-cyclopropyl-7-(2,8-diazabicyclo[4.3.0]nonane)-6-fluoro-8-chloro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid hydrochloride (I, Fig. 1), is a novel 4-quinolone with outstanding antimicrobial activity against Gram-negative and Gram-positive pathogens as well as anaerobic and intracellular bacteria and might be an important contribution to progress in anti-infective therapy. Clinical development of I requires a specific and sensitive method for quantification of the drug in human biological material (*e.g.* plasma, urine, saliva) in order to gain information about the pharmacokinetics of the drug. Plasma concentrations of I of 0.02 up to 2 mg/l are regarded as clinically relevant and should be obtained with oral single doses of 50–200 mg.

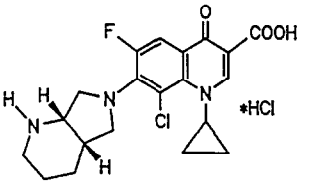

Fig. 1. Structure of BAY y 3118 (I).

Compound I itself exhibits no major fluorescence properties, whereas compounds with simi-

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lar structure, such as ciprofloxacin, exhibit strong fluorescence. However, the drug is slightly light-sensitive and the decomposition products show strong fluorescence properties. This fact was used to develop a sensitive HPLC method using on-line photo- or photothermal post-column derivatization and fluorescence detection. This technique has been found to be extremely useful for converting non-responding or poorly responding analytes to derivatives that can be detected with increasing sensitivity or specificity, together with either fluorescence [1,2] or electrochemical detection [3–9].

Photothermal post-column derivatization has already been successfully applied to compounds with similar structure to I [2]. For the decomposition process two different photochemical reactors were tested: (a) the commercially available Beam Boost photochemical reaction unit, which allows the photolysis time to be changed by using different lengths of capillaries, and (b) a modified laboratory-made device according to Scholl *et al.* [2], which also allows the eluent temperature to be changed.

Both systems were tested and the method was applied to determine plasma and urine concentrations of I after administration of 100 mg of the drug to a healthy male volunteer.

EXPERIMENTAL

Reagents

All reagents were of analytical grade and obtained from Merck (Darmstadt, Germany). BAY y 3118 (I) (Bayer, Leverkusen, Germany) was used as standard substance for the quantitative analysis (Lot R37-1). Since I is a hydrochloride (MW 442), but is found in biological fluids as the betaine (MW 405.5), all concentration calculations were based on the betaine.

Instrumentation

The HPLC system consisted of an HP 1090 liquid chromatograph with a temperature-controlled autosampler (HP 79847B) and an HP DOS Chem-station (HP G1304) (Hewlett Packard, Waldbronn, Germany) and a Spectroflow 980 fluorescence detector (Kratos, Weiterstadt, Germany).

The autosampler temperature was adjusted to 6°C using a Haake D8 water bath (Haake, Karlsruhe, Germany). The stationary phase was Nucleosil 100 C_{18} (5 μm particle size, 250 mm × 4.6 mm I.D.) with a guard column of the same material (M. u. W. Chromatografietechnik, Berlin, Germany). The mobile phase consisted of acetonitrile (19%, v/v) and an aqueous solution (81%, v/v) prepared as follows: 500 ml of 0.1 *M* tetrabutylammonium bromide solution were mixed with 500 ml of 0.05 *M* phosphoric acid and adjusted to pH 2.0. Before use the mobile phase was filtered through a 0.45-μm filter. The flow-rate was 1.0 ml/min and the oven temperature was 50°C. For fluorescence detection an excitation wavelength of 277 nm and an emission cut-off filter (418 nm) was used.

For the post-column derivatization either the Beam Boost photochemical reaction unit (ICT, Frankfurt, Germany) with a 20-m PTFE capillary and a GTE Sylvania G8T5 254-nm UV lamp or the laboratory-made device as described by Scholl *et al.* [2] was used. The principle of the laboratory-made device is as follows. The column eluate passes through a stainless-steel capillary in which it is heated by an adjustable heating element, and then into a 10-cm PTFE capillary (I.D. 0.3 mm) in which it is irradiated by a high-pressure mercury lamp (HPK 125 W, Philips Licht, Cologne, Germany), through a cooler and hence to the fluorescence detector. The lamp and PTFE capillary are enclosed by a single-walled nickel-plated brass cylinder of 55 mm I.D. The water-cooling system as described by Scholl *et al.* [2] was replaced by air cooling using a ventilator on top of the brass cylinder. The lamp gives a continuous spectrum with maxima at 270 and 313 nm and additional emission lines at 254/8, 313 and 366 nm.

Sample preparation

A 0.5-ml volume of plasma was diluted with 0.5 ml of 0.05 *M* phosphoric acid. For concentrations above 2.5 mg/l, 0.2 ml of plasma were diluted with 0.8 ml of 0.05 *M* phosphoric acid. The

samples were then centrifuged in closed, brown glass HPLC vials (No. 70214, Marcherey-Nagel, Düren, Germany) for 10 min at 1500 *g*. Saliva was collected using special test tubes (Salivette, No. 511539, Sarstedt, Nünbracht, Germany). After centrifugation, saliva was directly injected into the HPLC system.

For the quantification of I in urine, samples were diluted 1:50 up to 1:1000 with 0.1 *M* phosphate buffer pH 7.5, depending on urine concentrations of I. Injection volume was 20 μl for all biological fluids.

Calibration

For quantification, the external standard method was employed. Plasma calibration samples were obtained by adding 0.05 ml of a I working solution (concentrations ranging from 0.2 to 100 μg/l in 0.1 *M* phosphate buffer pH 7.5) to 0.95 ml of human blank plasma. A 0.5-ml aliquot of this mixture was placed in a brown HPLC vial and diluted with 0.5 ml of 0.05 *M* phosphoric acid and centrifuged for 10 min at 1500 *g*.

Urine calibration samples were obtained by adding 0.05 ml of I working solutions (concentrations ranging from 1 to 100 mg/l) to 0.95 ml of human blank urine diluted 1:100 with 0.1 *M* phosphate buffer pH 7.5

Calibration curves were obtained after analysing calibration samples of at least six different concentrations and plotting absolute peak height for I against the concentrations of I added (calculated as the betaine). Linear regression was performed using $1/y^2$ as a weighting factor. For unknown or other spiked samples the concentration of I was calculated using the regression function.

Assay validation

Intra-day variability. Five spiked samples of each concentration level (0.01, 0.02, 0.1, 1, 2 mg/l for plasma and 1, 25, 50, 100, 500 mg/l for urine) were analysed within one day using one calibration curve.

Inter-day variability. On five consecutive days one sample of each concentration level was analysed using one calibration curve per day.

Stability of I in plasma and urine

Human blank plasma was spiked with I to achieve concentrations of 0.05 and 0.2 mg/l. The spiked plasma was filled up in 1- and 5-ml portions and stored at −20°C. After 2, 5, 7 and 8 days and (only for 0.2 mg/l) after 9, 13 and 15 weeks one 1-ml sample was thawed and assayed. In addition, the 5-ml samples of each concentration level were thawed, 1 ml was taken for analyses and the remaining 4 ml were refrozen. This cycle was repeated four times. A urine sample (0.1 mg/l, pH 7) was stored for 24 h (12 h daylight, 12 h dark) in a glass tube and concentrations of I were determined at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h.

Application

One volunteer received 100 mg of I as part of a phase I study approved by an ethics committee and blood samples were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 48 h after administration and urine was collected up to 72 h.

RESULTS AND DISCUSSION

Chromatography

The chromatographic system used was a modification of the conditions described by Scholl *et al.* [2] for ciprofloxacin. Tetrabutylammonium bromide was used instead of the bisulphate salt, and different ratios of aqueous and organic phases were chosen. The salts in the mobile phase were necessary to prolong column life time by prevention of protein precipitation and not for the formation of ion pairs, because I and ciprofloxacin were positively charged at pH 2 [10]. About 2000 plasma samples could be analysed without loss of separation capacity, when the guard column was changed after every 100 samples.

Using the described chromatographic system with the laboratory-made photothermal post-column derivatization unit, compound I could be detected at a retention time of about 5.3 min (Fig. 2).

Possible metabolites of I known from preclinical investigations (acylglucuronide, N-sulphate

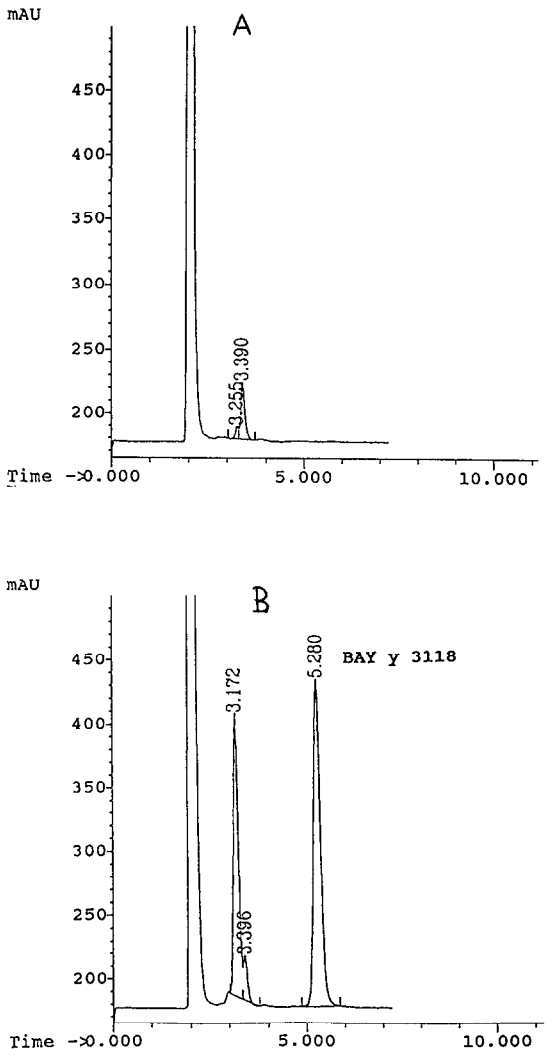

Fig. 2. Chromatogram of (A) blank plasma and (B) a 1-h plasma sample from a volunteer receiving 100 mg of I (concentration 0.59 mg/l) (retention time = 5.3 min).

conjugate) as well as endogenous compounds from plasma or urine do not interfere. The acyl-glucuronide could be detected in the described system with a retention time of about 3.2 min, whereas the N-sulphate conjugate was not detectable in this system. Peak heights of the I signal correlated linearly ($r > 0.99$) with I concentrations in the range 0.01–2.0 mg/l (plasma) and 1–500 mg/l (urine). The slope and intercept (mean ± S.D.) were calculated as 222.3 ± 3.3 and 204 ± 433.4, respectively. Tables I and II show the intra- and inter-day variability of the method for plasma and urine samples.

TABLE I

INTRA- AND INTER-DAY VARIABILITY (PLASMA SAMPLES)

Concentration added (mg/l)	Mean found concentration (mg/l)	C.V. (%)	Accuracy (%)
Intra-day			
2	2.15	4.03	7.6
1	1.08	3.31	7.6
0.1	0.11	5.11	5.7
0.02	0.02	3.47	−1.0
0.01	0.01	5.10	1.5
Inter-day			
2	2.05	2.64	2.3
1	1.02	2.74	2.1
0.1	0.10	2.84	2.2
0.02	0.02	9.76	4.7
0.01	0.01	8.20	5.9

The intra-day coefficients of variation ranged from 3.3 to 5.1 and 0.5 to 3.8% for plasma and urine samples, respectively. The inter-day coefficients of variation were slightly higher, ranging

TABLE II

INTRA- AND INTER-DAY VARIABILITY (URINE SAMPLES)

Concentration added (mg/l)	Mean found concentration (mg/l)	C.V. (%)	Accuracy (%)
Intra-day			
500	503.2	1.48	0.6
100	97.5	1.45	−2.5
50	49.9	0.49	−0.3
25	25.6	1.37	2.4
1	1.0	3.80	−2.8
Inter-day			
500	505.7	3.54	1.1
100	100.4	2.54	0.4
50	50.8	3.52	1.6
25	25.3	3.73	1.3
1	1.1	6.93	4.7

from 2.6 to 9.8% (plasma) and 2.5 to 6.9% (urine). The deviations of the assayed concentrations from the spiked concentrations (accuracy) were always less than ± 8%.

Post-column derivatization

The use of the laboratory-made photothermal derivatization unit offers several advantages compared with the Beam Boost reaction unit. The combination of photolysis and thermolysis permits the simultaneous changing of photolysis conditions (*e.g.* length of the PTFE capillary) and thermolysis conditions (*e.g.* the eluent temperature) without influencing the chromatographic performance.

The Beam Boost reaction unit allows only a change in photolysis conditions and requires long PTFE capillaries (5–20 m) in comparison with the 10-cm capillary used in the laboratory-made device. Therefore, retention time and peak shape will be influenced, if derivatization conditions are changed. A capillary length for the Beam Boost reaction unit of 20 m was associated with an increase in retention time of 2 min using a flow-rate of 1 ml/min, resulting in slightly broader peaks. The total way through the laboratory-made device was about 2 m (approximately 12 s). The time for photolysis was less than 0.6 s and for heating about 9 s. Fig. 3 demonstrates the influence of temperature and irradiation on the signal intensity for the laboratory-made device. With increasing temperature the signal became larger. A temperature of 275°C for the heating element was the optimum with respect to signal intensity, stability of the capillary and the chromatographic system. Only heating (without irradiation) was not able to decompose I to fluorescence compounds in a larger quantity. Only irradiation (without heating) resulted in a five-fold increase in peak height. Compared with the signal without heating and irradiation, an approximately 80-fold increase in signal intensity was observed using optimal conditions (275°C, irradiation).

Fig. 4. illustrates for the Beam Boost reaction unit the change in signal intensity with increasing irradiation time (increase in capillary length). The highest signal was obtained using a 20-m PTFE capillary, which resulted in a similar peak height as that obtained with the laboratory-made device. However, a further increase in capillary length resulted in a decrease of signal intensity, which might be explained by further decomposition to non-fluorescent compounds.

Stability of I in plasma and urine

Compound I was stable in frozen plasma (−20°C) over a period of at least 15 weeks. Also, repeated thawing and freezing had no influence

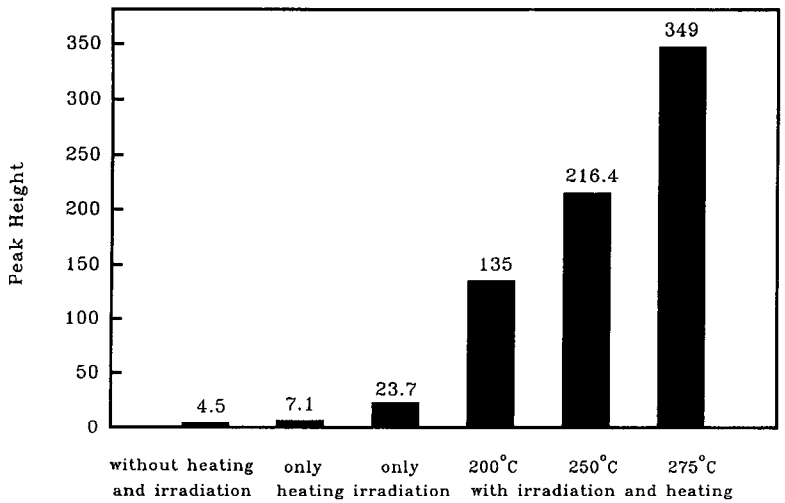

Fig. 3. Influence of temperature and irradiation on the fluorescence signal (laboratory-made device).

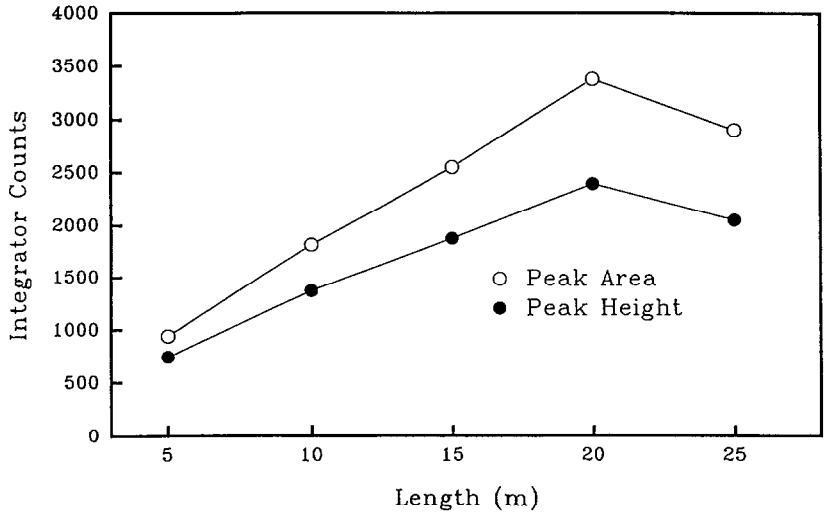

Fig. 4. Influence of capillary length on the fluorescence signal (Beam Boost reaction unit).

on I stability in plasma. In addition, I in urine (pH 7) was stable when stored in a glass tube at room temperature for 24 h (12 h daylight, 12 h dark).

Application

As part of a phase I clinical study, one volunteer received an oral dose of 100 mg of I. Plasma and urine concentrations were measured using the described HPLC method with the laboratory-made derivatization device. As shown in Fig. 5 maximum I plasma concentrations of 0.59 mg/l were reached 1 h after administration. A terminal half-life of 12 h was calculated. Plasma concentrations could be detected up to 48 h. About 9.3% of the dose was recovered in urine as unchanged I. Urine concentrations ranged from 1 to 9.5 mg/l (Fig. 6).

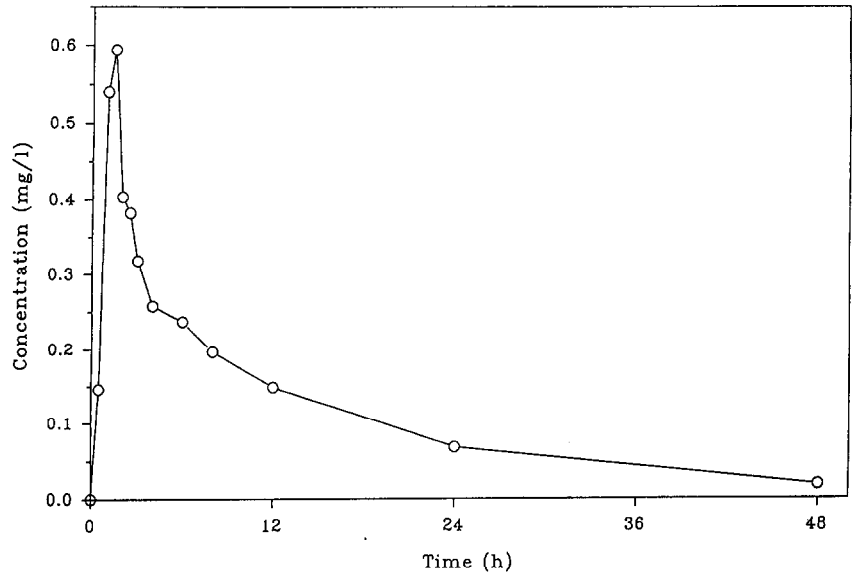

Fig. 5. Plasma concentration of I *versus* time profile in a healthy male volunteer after administration of a single 100-mg dose.

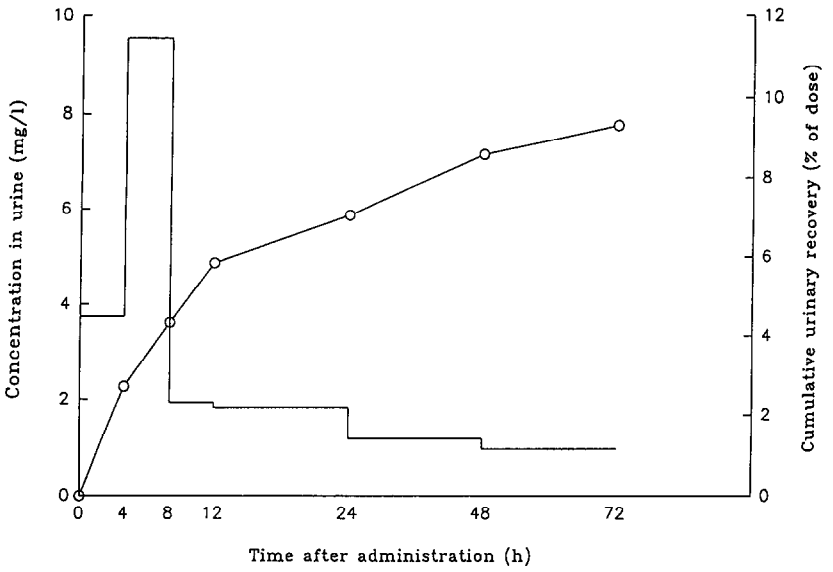

Fig. 6. Urinary excretion of I after administration of a single 100-mg dose in a healthy male volunteer.

CONCLUSION

A rapid and sensitive chromatographic procedure has been developed and extensively validated in order to quantify picogram amounts of I in biological fluids. The method utilized a post-column derivatization system that allows the simultaneous change of temperature and irradiation. The method was applied to determine the pharmacokinetic behaviour of I in a healthy volunteer and is currently used during phase I development of this compound.

REFERENCES

1 P. J. Harman and G. L. Blackman, *J. Chromatogr.*, 225 (1981) 131.
2 H. Scholl, K. Schmidt and B. Weber, *J. Chromatogr.*, 416 (1987) 321.
3 L. Dou, A. Holmberg and I. S. Krull, *Anal. Biochem.*, 197 (1991) 377.
4 W. L. Childress, I. S. Krull and C. M. Selavka, *J. Chromatogr. Sci.*, 28 (1990) 76.
5 L. Dou and I. S. Krull, *J. Chromatogr.*, 499 (1990) 685.
6 W. J. Bachman and J. T. Stewart, *J. Chromatogr. Sci.*, 28 (1990) 123.
7 N. D. Danielson and M. A. Targove, *J. Chromatogr. Sci.*, 26 (1988) 362.
8 M. Righezza, M. H. Murello and A. M. Siouffi, *J. Chromatogr.*, 410 (1987) 145.
9 C. M. Selavka, I. S. Krull and I. S. Lurie, *J. Chromatogr. Sci.*, 23 (1985) 499.
10 F. Sörgel, P. Muth, G. Mahr and M. Manoharan, in D. Adam (Editor), *FAC Advances in Antimicrobial-Antineoplastic Chemotherapy*, 6-10, Futuramed Publishers, Munich, 1987, p. 1963.