ELSEVIER

Journal of Chromatography A, 846 (1999) 175–180

JOURNAL OF
CHROMATOGRAPHY A

Short communication

Determination of trovafloxacin in human body fluids by high-performance liquid chromatography

Klaus Borner[a,*], Hildegard Hartwig[a], Hartmut Lode[b]

[a]Institute for Clinical Chemistry and Pathobiochemistry, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, D-12200 Berlin, Germany

[b]Department of Pneumology I, City Hospital Zehlendorf-Heckeshorn, Freie Universität Berlin, Zum Heckeshorn 23, D-14109 Berlin, Germany

Abstract

For the quantitative determination of trovafloxacin (a new naphthyridinone antibacterial agent) in serum and urine a simple isocratic HPLC method with fluorimetric detection is described. Serum was deproteinised with a mixture of acetonitrile and perchloric acid. The protein-free extract was separated on a reversed-phase column (Nucleosil 100-5 C_{18}) and quantified by means of fluorescence (excitation 275 nm, emission 405 nm). The mobile phase consisted of a mixture of 250 ml acetonitrile and 750 ml distilled water containing 10 mmol/l tetrabutylammonium phosphate. Urine was diluted with 0.25 mol/l phosphoric acid 1:20 (v/v) which was adjusted to pH 3.6 with sodium hydroxide solution. Diluted urine samples were separated on a cation-exchange column (Nucleosil 100-5 SA) and also detected by means of fluorescence. Trovafloxacin was sufficiently separated from endogenous compounds. Results of validation are given. The method was applied successfully to a study of healthy volunteers. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Trovafloxacin

1. Introduction

Trovafloxacin (**I**) is a new naphthyridinone ((1α,5α,6α)-7-(6-amino-3-azabicyclo[3.1.0]hex-3-yl)-1-(2,4-difluorophenyl)-6-fluoro-1,4-dihydroxy-4-oxo-1,8-naphthyridine-3-carboxylic acid) of the class of bacterial gyrase inhibitors (Fig. 1). The drug has high antimicrobial potency against many pathogenic species. For pharmacokinetic studies in humans after intravenous administration of the prodrug alatrofloxacin a sensitive and specific method of determination for trovafloxacin is required.

*Corresponding author. Tel.: +49-30-8445-2514; fax: +49-30-8445-4152.

2. Experimental

2.1. Materials

Trovafloxacin mesylate (lot No. 25391-070-04, potency 814 mg free base per gram) was kindly provided by Pfizer (Groton, USA). Analytical grade acetonitrile (Fisher Scientific, Wiesbaden, Germany), conc. perchloric acid, phosphoric acid, and sodium hydroxide (E. Merck, Darmstadt, Germany). Tetrabutylammonium hydrogensulfate, 5 mmol per bottle (PIC A reagent) (Waters, Eschborn, Germany). Blank serum (German Red Cross), blank urine from healthy volunteers. Double distilled water was used in all experiments.

0021-9673/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00247-2

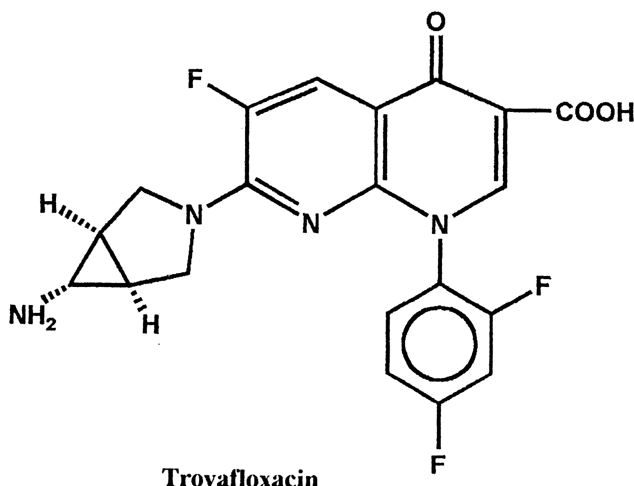

Fig. 1. Chemical structure of trovafloxacin.

2.2. Solutions

Solution A: dilute 0.5 ml conc. perchloric acid in 200 ml acetonitrile.

Solution B: dissolve 10 mmol tetrabutylammonium hydrogensulfate in 1000 ml distilled water. Adjust to pH 3.68 with ≈1.5 ml conc. phosphoric acid.

Solution C, mobile phase for serum: dissolve 10 mmol tetrabutylammonium hydrogensulfate in 700 ml distilled water. Add 250 ml acetonitrile. Adjust to pH 3.68 with ≈1.5 ml conc. phosphoric acid. Fill with distilled water to make 1 l. Filter through a 0.45 μm filter (type HVLP, Millipore, Eschborn, Germany).

Solution D: dissolve 3.2 g sodium hydroxide and 6.74 ml conc. phosphoric acid in 950 ml distilled water, adjust to pH 2.80 and fill with distilled water to make 1 l.

Solution E: dilute solution D with an equal volume of distilled water. Final pH 3.60.

Solution F, mobile phase for urine: mix 622 ml acetonitrile with 320 ml solution D, adjust to pH 3.60, fill to 1 l with solution D.

2.3. Calibrator and control samples

A stock standard solution was prepared by dissolving 30.71 mg trovafloxacin mesylate in 25 ml distilled water to give a trovafloxacin concentration of 1000 mg/l. It was stored at −75°C. The stock standard solution was diluted with drug-free serum to final concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 5.0, 7.5, and 10.0 mg/l. Urine standards were prepared from the stock standard solution with drug-free urine to final concentrations 0.6, 1.25, 2.5, 5.0, 12.5, 18.5, 37.5, and 50.0 mg/l. Control sera and urines were independently spiked in the same manner. For control of precision, additional serum pools from volunteers were collected.

2.4. Samples and storage

Serum and urine samples were obtained from 12 healthy volunteers who received a single i.v. dose of 300 mg alatrofloxacin (=200 mg trovafloxacin) within a pharmacokinetic study. All samples, calibrators and controls were stored without additives in closed polypropylene containers at −75°C. All samples were analysed within 6 weeks of sampling.

2.5. Sample pre-treatment

2.5.1. Serum

To 0.2 ml serum or calibrator, 0.4 ml solution A was added. The mixture was shaken for 3 min in a closed Eppendorf vessel and subsequently centrifuged for 3 min at 13 000 *g*. Supernatant (0.2 ml)

was transferred to an autosampler vial and diluted with 0.8 ml solution B.

2.5.2. Urine

Urine or calibrator (0.05 ml) was diluted with 0.95 ml solution E. Prepared samples are stable in the autosampler compartment at 10°C for at least 20 h (data not shown).

2.6. Chromatography and data handling

The chromatograph consisted of the following modules: a Model 510 isocratic pump (Waters), a Model 717 autosampler (Waters), a Model 470 fluorescence detector (Waters), and a Model 3396A integrator (Hewlett-Packard, Böblingen, Germany). Two different separation principles were applied for serum and urine. For serum, reversed-phase chromatography on a Nucleosil 100-5 C_{18} column (125 mm×4 mm I.D., particle size 5 μm, Macherey–Nagel, Düren, Germany) was used. For urine, cation-exchange chromatography using a Nucleosil 100-5 SA column (125×4 mm I.D., particle size 5 μm, Macherey–Nagel) was used. Both columns were protected by a guard column filled with Perisorb RP18 (30 mm×4 mm I.D., particle size 30–40 μm, E. Merck). All columns were operated at room temperature, i.e. ≈22°C. Flow-rates and pressures were 0.9 ml/min and 8.3 MPa for serum (solution C) and 1.0 ml/min and 12.0 MPa for urine (solution F). Injection volumes were 30 μl (serum) and 15–30 μl (urine). The fluorescence detector was operated at 275 nm for excitation and at 405 nm for emission. For calculation of linear calibration curves and concentrations peak heights were used. Typical retention times for **I** were 3.3 min (serum) and 7.0 min (urine). Total run times were 20 min (serum) and 10 min (urine).

2.7. Validation of the method

For calculation of validation data standard statistical calculations and a commercial software package were used (SQS, release 2.0, Perkin-Elmer, Überlingen, Germany).

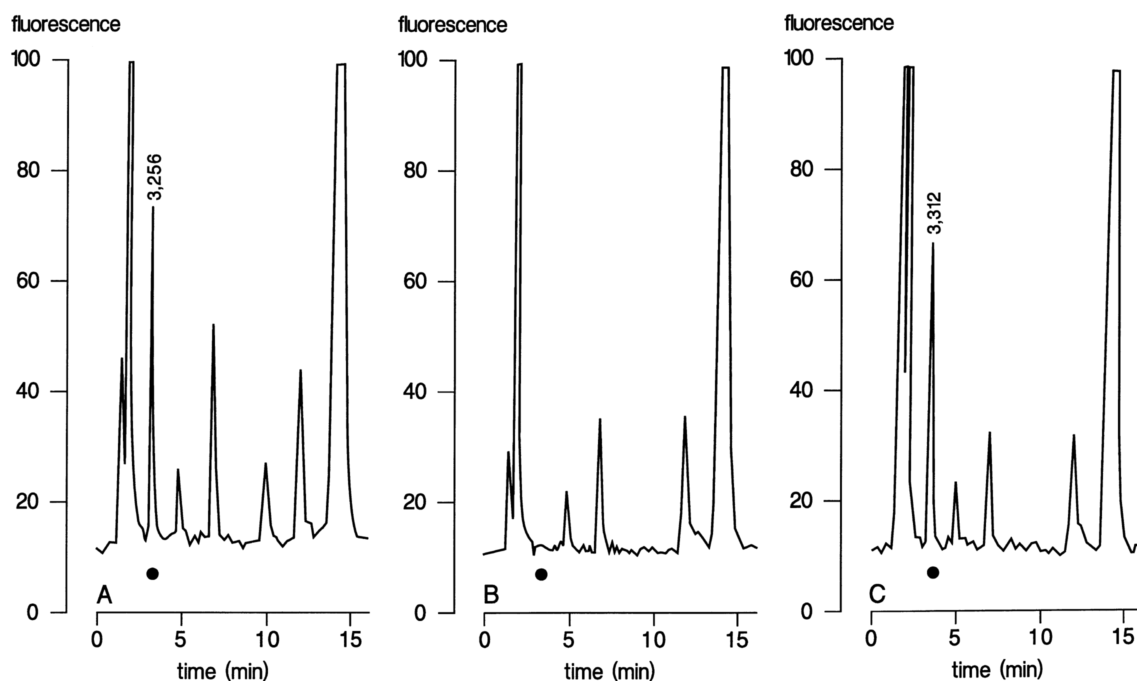

Fig. 2. Chromatograms from a blank serum spiked with 2 mg/l (A), a blank serum of a healthy volunteer (B), and the serum of a volunteer 4 h after an i.v. dose of 200 mg trovafloxacin, concentration 1.89 mg/l (C). The dot indicates the position of **I**.

3. Results and discussion

Compound **I** is a typical gyrase inhibitor, most of which are either fluoroquinolones or fluoronaphthyridinones. They have a characteristic UV absorbence spectrum and may show, depending upon the substituents, typical fluorescence spectra with a remarkable Stokes' shift of more than 100 nm. Compound **I** has both properties. The aim of our study was the development of a simple and specific analytical method for **I** in pharmacokinetic studies when other antibiotics, for example cefepime, were administered simultaneously. The only HPLC method published so far for **I**, by Teng et al. [1], uses solid-phase extraction and reversed-phase chromatography with UV detection. Unfortunately, our blank sera contained many interferences in the UV mode.

By using fluorescence detection instead of UV absorption, sufficiently clean chromatograms were obtained in reversed-phase chromatography of serum samples. Representative chromatograms are shown in Fig. 2. With urine samples a far better separation of **I** was achieved by cation-exchange chromatography. For typical chromatograms, see Fig. 3. Urine chromatograms showed at least one potential metabolite of **I** with a retention time slightly shorter than the parent compound. Identification of the metabolite was not possible at the time of the investigation when metabolites of **I** in humans or animals were unknown. Recent results in rats and dogs indicate that glucuronidation and *N*-acetylation may be major pathways of biotransformation of **I** [2,3]. The prodrug of **I**, alatrofloxacin, could not be detected in serum of human volunteers after i.v. infusion, probably due to rapid conversion to **I**.

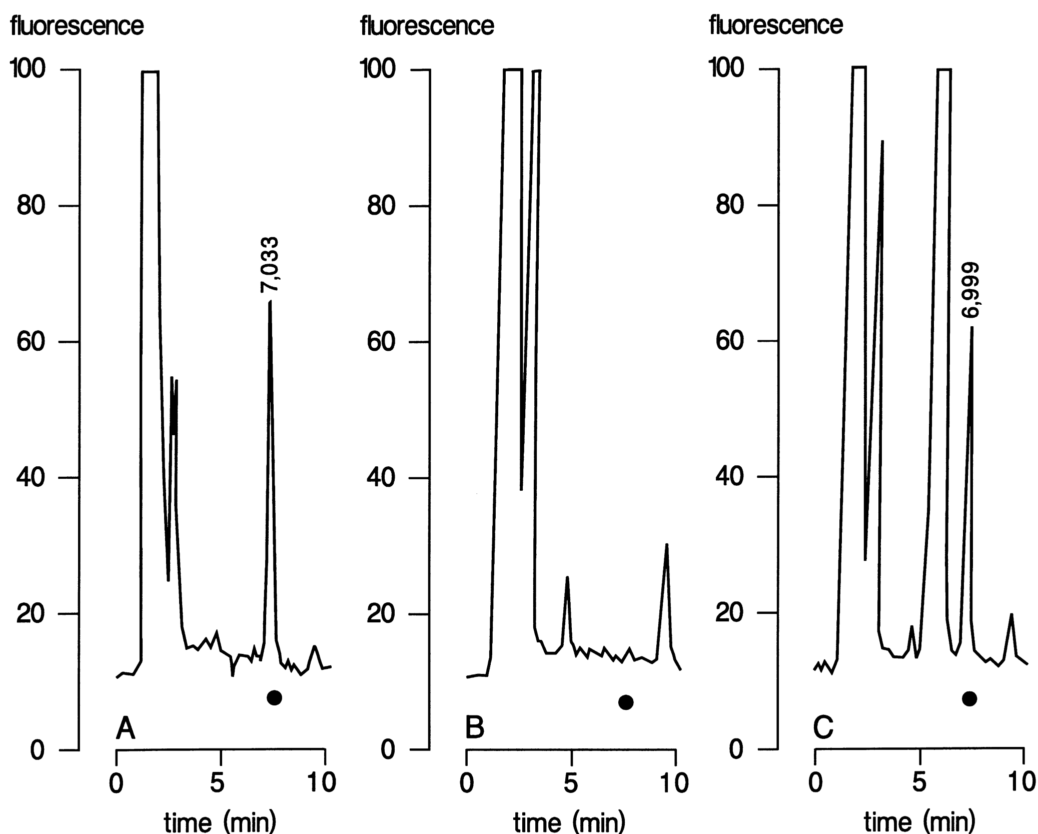

Fig. 3. Chromatograms from a blank urine spiked with 12.5 mg/l (A), a blank urine from a healthy volunteer (B), and urine collected 3 to 6 h after 200 mg trovafloxacin i.v., concentration 11.3 mg/l (C). The dot indicates the position of **I**.

Table 1
Validation of the method

	Serum	Urine
Detection limit (mg/l)	0.02	0.1
Lower limit of quantification (mg/l)	0.07	0.5
Linear range (mg/l)	7.5	50
Correlation coefficient (*r*)	≥0.9999	≥0.9999
Precision within series (RSD, %)	2.6–10.1	1.1–2.0
Precision between series (RSD, %)	5.0–12.5	2.2–9.8
Recovery (%)	98–108	99.6–107.2
Interference	None in healthy volunteers	

Use of fluorescence detection, which is more specific for quinolones than UV detection, allowed a simple and fast sample preparation by means of precipitation of serum proteins. This procedure is also rather cheap compared to solid-phase extraction.

The results of an extensive evaluation of the method are summarised in Table 1. Detection limits, lower limits of quantification, and correlation coefficients were calculated from four and six calibration curves for serum and from seven calibration curves for urine. These parameters were calculated for a confidence interval of 95%. Peak heights and peak areas yielded identical limits of detection and lower limits of quantification for serum (data not

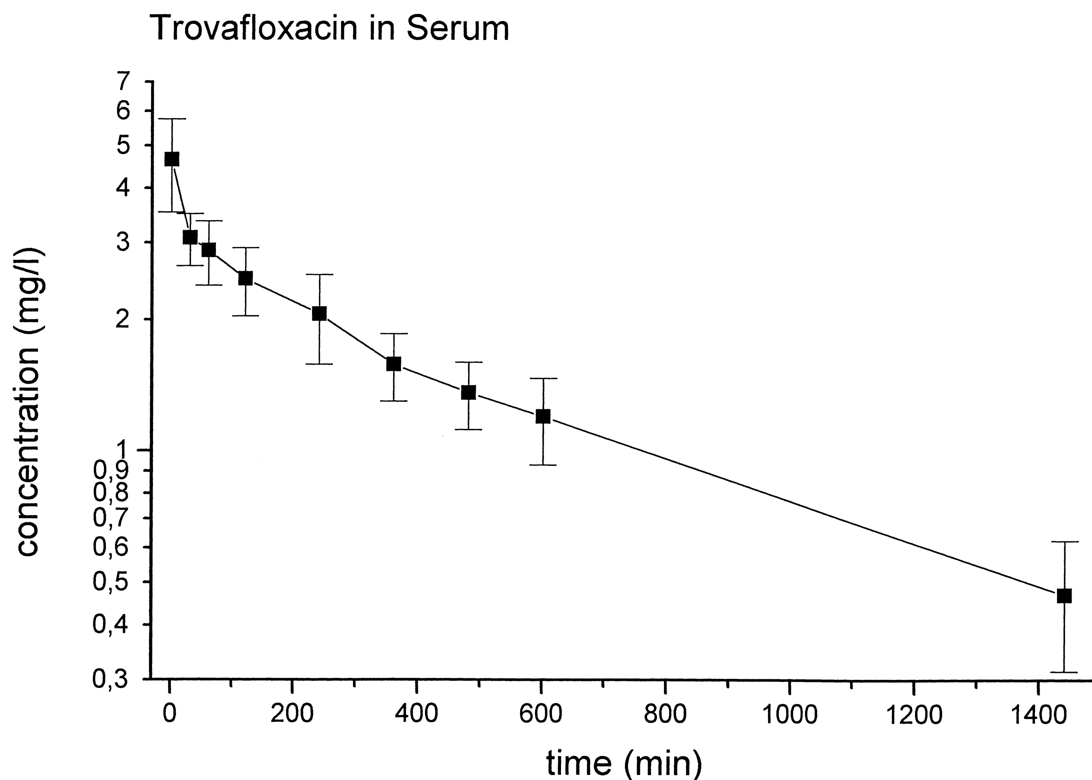

Fig. 4. Serum concentrations (mean±SD) of trovafloxacin in 12 healthy volunteers after a single intravenous dose of 300 mg alatrofloxacin (equivalent to 200 mg trovafloxacin).

shown in Table 1). Calibration curves were linear within the quoted range of concentrations. Precision within series was determined in four spiked sera having concentrations from 0.5 to 3.2 mg/l (n=10). Recovery in this series ranged from 98.1 to 108.0%. In another experiment, blank sera from 12 volunteers were spiked with 2 mg/l of **I**. The mean recovery was 98.0±2.3%. Precision between series varied between 5.0 and 12.5% in six control sera with concentrations between 0.5 and 3.2 mg/l. Precision within series measured in two urine pools was 1.1 and 2.2% (n=10; c=16.0 and 5.6 mg/l). Precision between series was determined in three urine samples (c=2.9, 8.0 and 16.0 mg/l). RSDs ranged from 9.8 to 2.2%. Recovery from spiked urine was 99.6 and 100.6% in this experiment. Mean recovery from 12 blank urines, spiked with 12.5 mg/l, was 107.2±4.6%. No interferences were observed in serum and urine from healthy volunteers.

The stability of **I** in serum was studied in nine serum samples that were stored at −75°C and then re-analysed. Original concentrations varied from 0.55 to 6.0 mg/l. After 18 months of storage, concentrations had fallen to 78.8±10.0% of the initial values.

The method was applied successfully to serum and urine samples from a study with healthy human volunteers. Mean serum concentrations vs. time are shown in Fig. 4.

References

[1] R. Teng, T.G. Tensfeldt, T.E. Liston, G. Foulds, J. Chromatogr. B, Biomed. Appl. 675 (1996) 53–59.
[2] D.K. Dalvie, N.B. Khosla, K.A. Navetta, K.E. Brighty, Drug Metab. Dispos. 24 (1996) 1231–1240.
[3] D.K. Dalvie, N. Khosla, J. Vincent, Drug Metab. Dispos. 25 (1997) 423–427.