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Determination of trovafloxacin, a new quinolone antibiotic, in biological samples by reversed-phase high-performance liquid chromatography

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Abstract

A simple, accurate and precise high-performance liquid chromatographic method was developed and validated for the determination of trovafloxacin, a new quinolone antibiotic, in serum and urine. Following solid-phase extraction, chromatographic separation was accomplished using a C_{18} column with a mobile phase consisting of 0.04 *M* H_3PO_4–acetonitrile–tetrabutylammonium hydroxide–0.005 *M* dibutyl amine phosphate (D-4) reagent (83:16.85:0.05:0.1, v/v), pH 3. Trovafloxacin and the internal standard (a methyl derivative of trovafloxacin) were detected by ultraviolet absorbance at 275 nm. The lower limit of quantification for trovafloxacin was 0.1 μg/ml and the calibration curves were linear over a concentration range of 0.1 to 20.0 μg/ml ($r^2 = 0.9997$). The average recoveries were greater than 70% for both trovafloxacin and internal standard. The intra-day and inter-day coefficients of variation were generally less than 5% in urine and serum over the concentration range of 0.1 to 20.0 μg/ml. Human serum samples could be stored for up to 12 months at −20°C and urine samples could be stored up to 18 months at −80°C.

Keywords: Trovafloxacin

1. Introduction

Trovafloxacin, 7-(3-azabicyclo[3.1.0]hexyl)-naphthyridone (CP-99,219), is a new synthetic fluoroquinolone antibiotic agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria (Fig. 1). It has shown a number of desirable characteristics in preclinical in vitro and in vivo testing. In broth susceptibility studies, trovafloxacin demonstrated a broad spec-

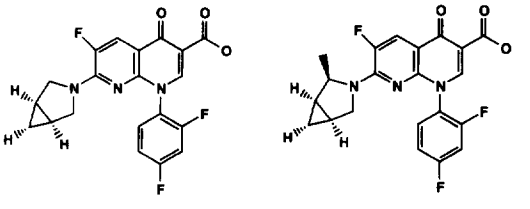

Fig. 1. Structures of trovafloxacin and the internal standard (CP-102,372).

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trum of in vitro activity against Gram-positive and Gram-negative bacteria and could be differentiated from ciprofloxacin, ofloxacin and other marketed fluoroquinolones by its greater potency against many clinically significant species of Gram-positive organisms, most notably against streptococci such as *Streptococcus pneumoniae* [1–4]. Trovafloxacin administered orally was able to control systemic infections of Gram-positive and Gram-negative organisms in mice; it was appreciably more potent than temafloxacin, ciprofloxacin and ofloxacin in protecting mice against lethal infections with *S. pneumoniae* or *Streptococcus pyogenes* [5].

The in vitro antimicrobial spectrum and potency of trovafloxacin justified further studies of its pharmacokinetics, metabolism and pharmacodynamics. A rapid, sensitive and precise analytical method for determination of trovafloxacin in serum and urine was essential for such investigations. The present study was undertaken to develop a rapid, specific reversed-phase HPLC assay for the determination of trovafloxacin in human serum and urine. The internal standard used in this assay is a methyl derivative of trovafloxacin (Fig. 1). The assay has been used to ascertain the stability of trovafloxacin in human serum and urine stored at −20° or −80°C. The method has been shown to be applicable to determination of trovafloxacin in human serum and urine.

2. Experimental

2.1. Chemicals and reagents

The mesylate salts of trovafloxacin and internal standard (I.S.), 7-(2-methyl-3-azabicyclo[3.1.0]hexyl)-naphthyridone, were synthesized at Pfizer Central Research Division (Groton, CT, USA). Potassium phosphate monobasic-sodium hydroxide buffer (pH 9.0), methanol and HPLC-grade acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tetrabutylammonium hydroxide and potassium phosphate monobasic were obtained commercially from Sigma (St. Louis, MO, USA). Dibutyl amine phosphate (D-4) reagent was bought from Waters (Milford, MA, USA), and 85% phosphoric acid and sodium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, USA). All chemicals were reagent grade and used as received without further purification. The water used in the preparations of reagent solutions and mobile phase was purified with a Milli-Q SP TOP system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of trovafloxacin were prepared by dissolving 3.28 mg mesylate salt of trovafloxacin in 25.0 ml of 0.025 *M* potassium phosphate monobasic solution, pH 3.0. This stock solution, equivalent to 100.0 μg/ml of trovafloxacin, was diluted to a standard solution of 10.0 μg/ml with the phosphate solution. The 10.0 μg/ml standard solution was further diluted to give an additional standard solution of 1.0 μg/ml. A stock solution of I.S. was prepared at a concentration of 100 μg/ml in 0.025 *M* potassium phosphate monobasic solution, pH 3.0. Standard solutions of I.S. were prepared by diluting the stock solution with the phosphate solution to 10 and 20 μg/ml. Both trovafloxacin and I.S. stock solutions and standard solutions were stored at 4°C and were stable for up to 2 months.

2.3. Chromatographic conditions

The HPLC system consisted of Model 600 solvent delivery system with a Model 717 automatic sample injector, and Model 486 ultraviolet absorbance detector and Model 600E system controller (Waters). The detector was operated at a wavelength of 275 nm. Chromatographic separation was accomplished using a Nova-Pak C_{18} analytical column (15 cm × 3.9 mm I.D., 4 μm particle size, Waters) and a Supelco Pelliguid LC-18 guard column (2 cm × 4.6 mm I.D., 4 μm particle size) with a mobile phase consisting of 0.04 *M* H_3PO_4–acetonitrile–tetrabutylammonium hydroxide–0.005 *M* dibutyl amine phosphate (D-4) reagent (83:16.85:0.05:0.1, v/v), pH 3. The mobile phase was delivered at a constant flow-rate of 0.6 ml/min for serum samples and 0.7

ml/min for urine samples at ambient temperature.

2.4. Serum and urine calibration standards and quality control samples

Calibration standards for the serum and urine assay were prepared by adding different volumes of trovafloxacin standard solutions to drug-free serum and urine. Serum calibration standards of trovafloxacin were prepared at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml. The urine trovafloxacin calibration standards were prepared at concentrations of 0.1, 0.3, 1.0, 3.0, 10.0 and 20.0 μg/ml. All serum and urine calibration standards were prepared daily. Quality control samples were independently prepared at concentrations of 0.3, 4.0 and 15.0 μg/ml in serum and 0.2, 2.0 and 15.0 μg/ml in urine. The quality control samples were labeled, dated, and frozen in 0.5 ml aliquots at −20°C for serum and 1.2 ml at −80°C for urine until the time of analysis.

2.5. Sample preparation

Serum

To a 200-μl aliquot of the blank, calibration standard, quality control sample or unknown human serum sample, 40 μl of the I.S. standard solution (10.0 μg/ml), and 0.5 ml of the 0.025 *M* (pH 3.0) phosphate solution were added, mixed well and applied to a C_{18} Polysorb MP-1 cartridge (Cat. No. 30100, Interaction Chemical, Mountain View, CA, USA), which was previously conditioned with 2 ml methanol and then 4 ml of the pH 3.0 phosphate solution. Using a Wheaton dispenser (Krackeler Scientific, Albany, NY, USA), the cartridge was washed with 4 ml of the pH 3.0 phosphate solution, and then the sample was eluted with 2 ml methanol. Each sample was then evaporated to dryness under a gentle stream of nitrogen gas in an analytical evaporator (Organomation, South Berlin, MA, USA) at 50–55°C. The resulting residue was reconstituted in 1.0 ml of the mobile phase and a 100-μl aliquot was injected onto the HPLC column.

Urine

To a 500-μl aliquot of the blank, calibration standard, quality control or unknown human urine samples, 100 μl of the I.S. standard solution (20 μg/ml) and 0.5 ml of 0.025 *M* (pH 3.0) potassium phosphate monobasic solution was added, vortex-mixed and applied to a bonded silica solid-phase extraction column (Cat. No. 1210-2051, Varian, Harbor City, CA, USA) which had been pre-treated with 3 ml methanol and 3 ml of the pH 3.0 phosphate solution, using a Wheaton dispenser at $<3 \times 10^{-3}$ kg/m^2 vacuum. The column was then rinsed with 3 ml pH 3.0 phosphate solution and then 3 ml methanol. The column was dried by full vacuum application for 2 min and transferred to a 16 × 100 mm culture tube. Elution was accomplished with 3 ml of pH 9.0 potassium phosphate monobasic-sodium hydroxide buffer–acetonitrile (25:75, v/v). The elution solvent was allowed to drip through the packing by gravity alone until it reached the top of the bed. The tube was then centrifuged for 2 min at 1000 *g*. The sample was evaporated to dryness under a gentle stream of nitrogen gas in an analytical evaporator at 50–55°C. The residue was reconstituted in 1.0 ml mobile phase and a 100-μl aliquot was injected onto the HPLC column.

2.6. Calibration and calculation procedure

Calibration curves were determined by a linear regression. A serum standard curve was constructed to relate the log-transformed peak-height ratios of trovafloxacin to the I.S. to the log-transformed standard concentrations of trovafloxacin, while a urine standard curve was generated using weighted linear regression to associate the peak-height ratios of trovafloxacin to the I.S. with the standard concentrations of trovafloxacin. The unknown concentrations of trovafloxacin in serum and urine samples were calculated by interpolation using these daily calibration curves.

3. Results and discussion

3.1. HPLC profiles

Adequate chromatographic separation was obtained using the system described above. Representative chromatograms of serum and urine are shown in Figs. 2 and 3. In a one-day validation, the retention times (mean ± S.D., $n = 6$) for trovafloxacin and I.S. are 5.542 ± 0.0175 and 6.678 ± 0.0301 min for serum samples and 6.889 ± 0.0170 and 8.300 ± 0.0258 min for urine samples. Fig. 2 shows the chromatograms from the analysis of blank human serum, the serum spiked with 0.1 μg/ml trovafloxacin and I.S., and the serum sample obtained at 4 h post-dose from a subject receiving a 100-mg oral dose of trovafloxacin, respectively. Fig. 3 illustrates the chromatograms from the analysis of blank urine, the urine spiked with 0.1 μg/ml trovafloxacin and I.S., and the urine samples collected at an interval of 0–24 h post-dose from a subject receiving a 100-mg oral dose of trovafloxacin, respectively. With the sample preparation procedure above, the blank chromatogram contained no peaks at the retention times corresponding to trovafloxacin or I.S.. Both peaks of interest were resolved from each other and from other components in the extracts. These suggest the lack of interference from endogenous compounds in either serum or urine.

3.2. Calibration curve and limit of quantification

The assays exhibited linearity ($r^2 > 0.9997$) over the 0.1 to 20.0 μg/ml ranges in serum (typical equation: $y = 0.99476x + 0.36805$) and in urine ($y = 2.95979x + 0.0060383$), where y is the peak-height ratio of trovafloxacin over the internal standard, and x is the trovafloxacin concentration (μg/ml). In practice, a series of standards was analyzed together with study samples, and these daily calibration curves were used for calibration and calculation purposes. In addition,

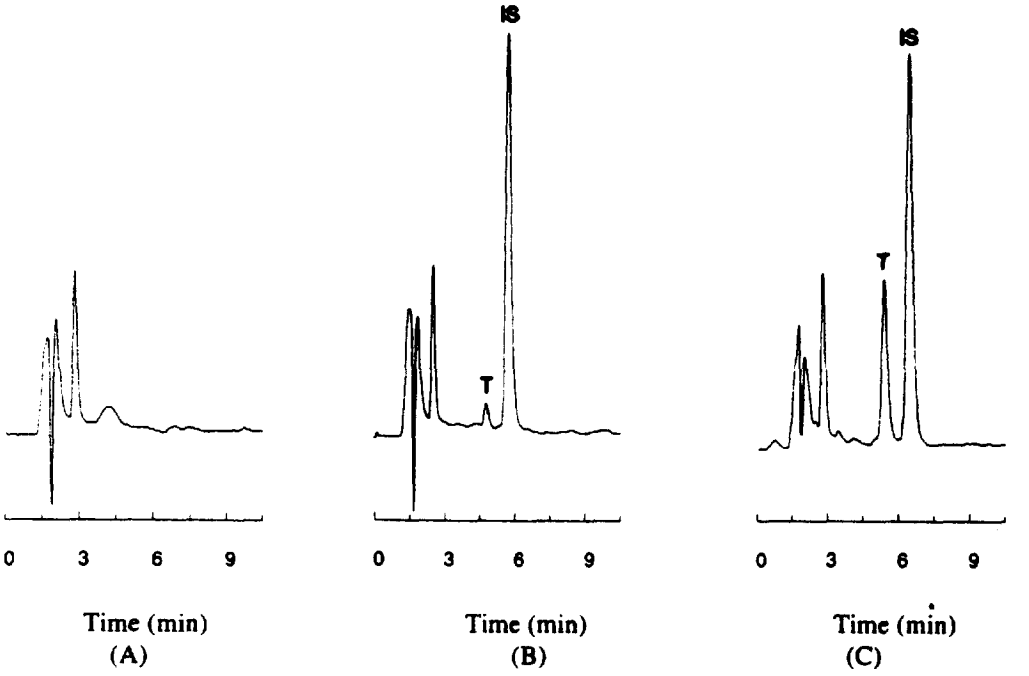

Fig. 2. The chromatograms resulting from the analysis of blank human serum (A), the serum spiked at 0.1 μg/ml trovafloxacin and I.S. (B) and the serum sample (0.7 μg/ml) obtained at 0.5 h post-dose from a subject who received a single 100-mg oral dose of trovafloxacin (C), respectively. Peaks: T = trovafloxacin; IS = internal standard.

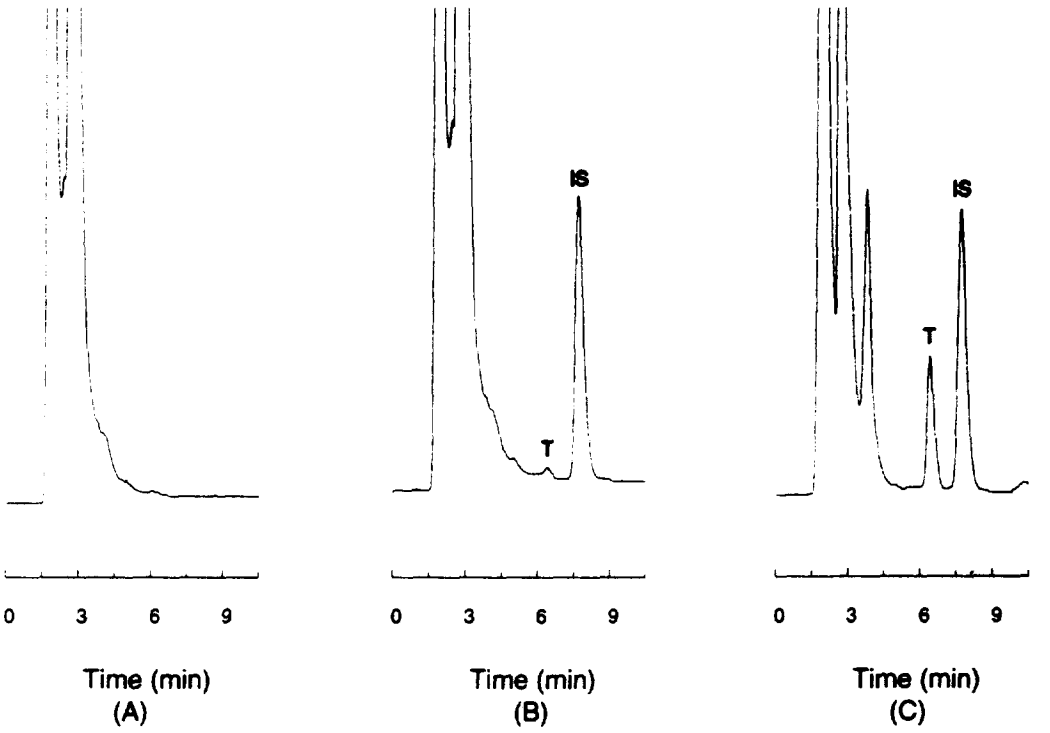

Fig. 3. The chromatograms resulting from analysis of blank urine (A), blank urine spiked with 0.1 μg/ml trovafloxacin and I.S. (B) and the urine sample (1.6 μg/ml) collected at an interval of 24–48 h post-dose from a subject who received a single 100-mg oral dose of trovafloxacin (C), respectively. Peaks: T = trovafloxacin; IS = internal standard.

quality control samples were analyzed to assure the accuracy and precision of daily analyses. Lower limit of quantification for trovafloxacin was assessed following the analyses of six low calibration standards on one validation day. The precision for the 0.100 μg/ml standard was 6.36% in serum and 5.25% in urine.

3.3. Recovery

The extraction recoveries of trovafloxacin and I.S. from human serum and urine samples were calculated from comparison of the peak heights of directly injected standards and those from serum or urine spiked with standards and submitted to the sample preparation procedures ($n = 6$). The extraction recoveries of trovafloxacin in serum at concentrations of 0.3, 4.0 and 15.0 μg/ml were 80.9, 71.8 and 74.0%, respectively. The extraction recovery of I.S. when added to serum to achieve a concentration of 2.0 μg/ml was 74.4%. The extraction recoveries of trovafloxacin in urine at concentrations of 0.200, 2.00 and 15.0 μg/ml were 71.9, 75.7 and 78.9%, respectively. The extraction recovery of I.S. when added to urine to achieve a concentration of 4.00 μg/ml was 78.7%.

3.4. Accuracy and precision

The accuracy and precision of the method were estimated from the back-calculated standard concentrations and by replicate analysis of quality control samples that were prepared independently. The results for the back-calculated serum and urine standards are shown in Table 1. The overall mean precision, as defined by the coefficient of variation (C.V.), ranged from 0.558% to 2.22% for serum samples with relative errors ranging from 0.0 to 2.0%. The C.V.s for urine samples ranged from 0.650% to 3.24% with relative error less than 1%. Analysis of the

Table 1
Accuracy and precision of the HPLC methods for the determination of trovafloxacin in human serum and urine

Concentration added (μg/ml)	Concentration found (mean ± S.D. $n = 5$) (μg/ml)	Coefficient of variation (%)	Relative error (%)
Serum			
0.1	0.101 ± 0.002	1.980	1.000
0.2	0.196 ± 0.004	2.041	−2.000
0.5	0.510 ± 0.008	1.569	2.000
1	0.994 ± 0.006	0.604	−0.600
2	1.974 ± 0.028	1.418	−1.300
5	5.024 ± 0.081	1.612	0.480
10	9.980 ± 0.142	1.423	−0.200
20	19.940 ± 0.148	0.742	−0.300
Urine			
0.1	0.100 ± 0.001	1.000	0.000
0.3	0.299 ± 0.010	3.344	−0.333
1	1.002 ± 0.022	2.196	0.200
3	2.980 ± 0.032	1.074	−0.667
10	10.030 ± 0.066	0.658	0.300
20	20.020 ± 0.130	0.649	0.100

independently prepared quality control samples are presented in Table 2. The mean intra-day and inter-day C.V.s for the serum sample analysis were 2.71% or less and relative errors were less than 2%. For the urine samples, the mean intra-day and inter-day C.V.s varied from 1.39% to 3.48% and relative errors ranged from 2.0% to 4.5%. The results indicated that these assays were reliable and reproducible.

3.5. Storage stability

Freeze–thaw stability of trovafloxacin was determined in serum and urine. The serum samples containing 0.200, 4.00 and 15.0 μg/ml of trovafloxacin were stored at −20°C and the urine samples containing 0.300, 4.00 and 15.0 μg/ml of trovafloxacin were stored at −80°C. After three freeze–thaw (room temperature) cycles the

Table 2
Statistical evaluation of the analysis results for trovafloxacin in quality control samples

Concentration (μg/ml)	Intra-day precision		Inter-day precision	
	(C.V., $n = 6$) (%)	Relative error (%)	(C.V., $n = 5$) (%)	Relative error (%)
Serum				
0.3	2.42	0.33	1.26	−0.33
4	1.81	−1.50	1.16	−1.75
15	2.71	0.00	1.21	−1.33
Urine				
0.2	3.48	−4.50	1.54	−4.50
2	1.39	−1.00	2.02	−2.00
15	1.62	−0.67	2.12	−2.00

trovafloxacin concentration was 98.4% to 99.3% of that obtained from the control matrixes (data not shown). The stability of trovafloxacin in the reconstituted serum or urine extracts was determined to be at least 24 h based on adequate precision and accuracy of the quality control results obtained during routine runs and the results of an autosampler tray satiability experiment. The concentration of trovafloxacin obtained in reconstituted serum or urine extracts incubated at room temperature for 24 h ranged from 98.0% to 100% of those obtained from the control extracts.

The storage stability of trovafloxacin in human serum and urine was evaluated at −20 and −80°C, respectively. The serum samples containing 0.200, 4.00 and 15.0 μg/ml of trovafloxacin were stored at −20°C and the urine samples containing 0.300, 4.00 and 15.0 μg/ml of trovafloxacin were stored at −80°C. Periodically, samples were removed from the freezer, equilibrated to room temperature and analyzed in replicate ($n = 6$) using freshly prepared serum and urine calibration standards. Results of the stability analysis indicate that trovafloxacin was stable at −20°C in frozen human serum for up to 12 months and at −80°C in urine for at least 18 months (Table 3).

Table 3
In vitro storage stability data for trovafloxacin in human serum and urine[a]

Storage time (days)	Nominal concentration (μg/ml) at −20°C in serum		
	0.200	4.00	15.0
1	0.0	−2.0	−2.7
6	6.0	−0.2	−3.0
32	2.0	−0.6	−1.3
43	7.0	−1.4	0.7
135	4.0	−1.9	1.0
225	2.0	−2.2	−0.3
365	−3.3	4.0	3.0
Storage time (days)	Nominal concentration (μg/ml) at −80°C in urine		
	0.300	4.00	15.0
1	−5.0	−4.5	−4.0
7	−4.5	−2.0	−1.3
21	2.0	−0.6	−1.3
43	7.0	−1.4	0.7
365	−18.7	1.0	0.0
540	−3.5	2.0	1.0

[a] Reported as the mean percentage of nominal concentration ($n = 6$).

In summary, the analytical method described is rapid, sensitive, selective and reproducible and has been used to study the pharmacokinetics of trovafloxacin in humans [6]. Without any modification, the procedure was also extensively used for pharmacokinetic studies in experimental animals [7].

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