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High-performance liquid chromatographic procedures for the determination of temafloxacin in biological matrices

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ABSTRACT

A simple and precise high-performance liquid chromatographic procedure has been developed for the determination of temafloxacin and its trace level metabolites in biological matrices. Plasma samples are ultrafiltered after addition of an internal standard in a displacing reagent containing sodium dodecyl sulfate and acetonitrile. Plasma ultrafiltrates or diluted urines are chromatographed on a reversed-phase analytical column, using an ion-pair chromatographic mobile phase and fluorescence detection. The chromatographic system allows resolution and quantitation of temafloxacin's oxidative metabolites, which collectively account for less than 2% of the administered dose. The mean intra-assay coefficient of variation for determination of temafloxacin concentrations in plasma ranging from 0.05 to 10.0 μg/ml was 0.7%. The procedure was implemented at four laboratories for the analysis of over 12 000 samples from clinical studies. Inter-assay coefficients of variation estimated from routine analyses of quality control samples in these studies averaged 4% or lower for concentrations in the 0.15–10 μg/ml range. The limit of quantitation of the procedure is approximately 10 ng/ml; inter-assay coefficients of variation at 15 ng/ml averaged under 9%. Calibration curves were reproducible and highly linear, with correlation coefficients typically averaging over 0.9995. An alternative, more complex procedure, involving methylene chloride extraction, which extends the detection limits to below 1 ng/ml, is also described.

INTRODUCTION

Temafloxacin (Abbott-63004) is a quinolone antibacterial with high activity against gram-positive and gram-negative bacteria (Fig. 1). In animals and man, the preponderant component in plasma and urine is unchanged temafloxacin; however, glucuronidation, with subsequent hepatobiliary secretion, also contributes to total clearance, moreso in animals than in man. Oxidation at the piperazinyl moiety is a minor pathway, typically accounting for less than 1–2% of the administered dose in man. Although bioassays provide a high throughput means of monitoring temafloxacin, they suffer disadvantages for pharmacokinetic and metabolic studies, in which precision, specificity and low quantitation limits are held at a premium. Since all recently developed quinolone antibacterials have both a carboxylic acid group and a strongly basic center, they are ionized throughout the pH range, thus affording challenges in work-up and chromatography. The most commonly selected approaches are deproteinization [1–3] or

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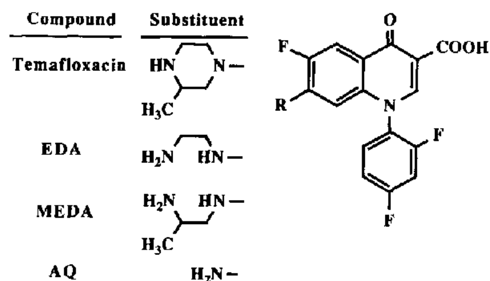

Fig. 1. Chemical structures of temafloxacin and its oxidative metabolites.

extraction with methylene chloride or chloroform [4–7], usually followed by reversed-phase high-performance liquid chromatography (HPLC) [1,3,5–9]. Recently, a procedure involving multiple extractions followed by reversed-phase HPLC has been proposed for the determination of temafloxacin in biological fluids [10]. Described herein is a simpler and more precise procedure for determination of temafloxacin and its metabolites in biological matrices.

EXPERIMENTAL

Chromatography

Plasma ultrafiltrates and diluted urines are chromatographed at ambient temperature on an Adsorbosphere HS C_{18} column (Alltech, Deerfield, IL, USA; 7-μm particles, 250 mm × 4.6 mm I.D.) using a mobile phase consisting of HPLC-grade acetonitrile–water (53:47) containing 0.04 *M* H_3PO_4, 0.01 *M* NaH_2PO_4, 0.2% sodium dodecyl sulfate (SDS) and 0.005 *M* N-acetylhydroxamic acid (Aldrich, Milwaukee, WI, USA). The primary detection mode for temafloxacin is fluorometric (Model FS970, Kratos, Ramsey, NJ, USA) with excitation at 280 nm and an emission cut-off of 389 nm.

Reference standards of temafloxacin and its oxidative metabolites as well as the internal standard were synthesized at Abbott Labs. (Abbott Park, IL, USA). Structures of temafloxacin and its oxidative metabolites are shown in Fig. 1. Analogous to most other quinolones, the metabolism of temafloxacin is very limited, involving conjugation and sequential oxidation of the methylpiperazine ring to produce ethylenediamine-substituted analogues (EDA, MEDA) and then an aminoquinolone (AQ).

Plasma procedures

Two procedures may be employed for the analysis of plasma samples. The primary procedure, which is superior in terms of precision and simplicity, involves ultrafiltration of plasma treated with a displacing reagent containing internal standard. The secondary procedure, which affords lower ultimate quantita-

tion limits with attendant increases in procedural complexity, involves extraction of plasma with a methylene chloride-ethanol (9:1) solvent system.

Temafloxacin is approximately 26% bound in human plasma; thus, the ultrafiltration procedure requires the use of a reagent capable of displacing temafloxacin from its binding sites. This displacing reagent consists of acetonitrile–water, containing 0.5% SDS and 0.075 *M* phosphate (pH 7.4) (30:70 v/v). On the day of use, the reagent is supplemented with internal standard (typically 0.1–0.5 μg/ml), thereafter to be dispensed by a high-precision device into disposable glass tubes (typically 0.4 ml in 75 mm × 10 mm tubes). Into these tubes equal volumes of plasma standards or unknowns are pipetted, and the contents are thoroughly mixed prior to transfer into an Amicon (Lexington, MA, USA) Centrifree™ apparatus, using "YMT" membranes. In accordance with manufacturer's specifications, the devices are centrifuged in a fixed-angle device for at least 20 min at relative centrifugal forces less than 1000 *g*. Under these conditions, typically more than 70% of the sample volume is ultrafiltered. Ultrafiltrates are assayed without further treatment.

Maintenance of high precision requires special attention in precise pipetting of samples and dispensing of displacer. With time, the acetonitrile in the displacer will degrade the ultrafiltration membrane; accordingly, after plasma and displacer are mixed, the samples should be ultrafiltered within 30 min.

In the extraction procedure, plasma (400 μl) is pipetted into 125 mm × 16 mm disposable glass tubes, followed by an equal volume of internal standard in 0.5 *M*, pH 7 phosphate buffer. The buffered plasma samples are then extracted with 6 ml of HPLC–Spectro-grade methylene chloride (Alltech) containing 10% ethanol for 10 min by slow-speed horizontal agitation (Eberbach, Ann Arbor, MI, USA). Other mixing procedures may be employed; however, care must be taken to prevent emulsion formation. After extraction and refrigerated centrifugation (10 min at 900 *g*) of the sample, aliquots of the organic phase are evaporated at 50–60°C under an air stream, and the residues are reconstituted in mobile phase for injection into the HPLC system.

Urine procedure

The levels of temafloxacin and its oxidative metabolites in urine are determined by direct injection of samples and standards which have been diluted in mobile phase (typically 1:20 to 1:100) and supplemented with internal standard (see Fig. 2). Approximately 60–70% of the dose administered to humans is excreted unchanged; thus, with the knowledge of urinary output, the range of the calibration curve and the appropriate dilutions can be determined from the dosage level. At dosage levels exceeding 600 mg, the equilibrium solubility of temafloxacin in cold urine may be exceeded; thus, samples which have been frozen should be incubated at 37°C and thoroughly mixed prior to dilution.

Temafloxacin and its oxidative metabolites (EDA, MEDA and AQ) are stable under short exposure to alkaline conditions; thus, incubating urine in 1.0 *M*

sodium hydroxide for 0.5 h at 60°C, followed by neutralization and dilution, provides a simple procedure to assess the extent of conjugation of parent and metabolites. Hydrolysis may also be accomplished by incubation with β-glucuronidase for 18 h at 37°C; however, acyl migration occurs in alkaline samples, resulting in recoveries slightly lower than those obtained by alkaline hydrolysis.

Preparation of calibration curves

For plasma, the initial stock solution (100 or 200 μg temafloxacin base per ml) is prepared in distilled water. This is diluted initially 1:10 into control plasma, followed by serial dilution with control plasma to provide standards down to 5 or 10 ng/ml. Preparation of standards in blank pooled urine is similar to that for plasma, except that the primary aqueous stock solution is prepared at 1–4 mg/ml. For both plasma and urine calibration curves, it should be noted that temafloxacin is usually supplied as the hydrochloride salt and that a proportionate excess must be weighed to account for HCL (~8% of the weight), surface water, and other trace components detected in the analytical assessment of that particular lot.

RESULTS AND DISCUSSION

Work-up procedures

Early experiments with temafloxacin and previous studies with its analogues have shown that extraction (liquid–liquid or disposable columns) and precipitation procedures cannot match the ultrafiltration technique in terms of simplicity and precision. Protein precipitation procedures are generally simple, but they either involve dilution or produce solutions that are considerably different in composition from the mobile phase, potentially resulting in chromatographic artifacts. Preliminary evaluation of acetonitrile and trichloroacetic acid as deproteinizing agents produced recoveries lower than 80%, apparently as a result of coprecipitation. Extraction procedures are more labor-intensive, tend to be less precise, and often do not recover polar metabolites. Typical intra-assay coefficients of variation (C.V.s) for the methylene chloride and chloroform extraction procedures are in the 3–5% range [4–7,9]. Microbiological procedures are generally the least precise and suffer the additional disadvantages of non-linear response curves, poor specificity, restricted detection limits, and matrix interactions. Accordingly, the ultrafiltration approach [9,11,12], when applicable, offers distinct advantages over the alternatives. Successful adaptation requires effective displacement of the analytes from plasma proteins and their chromatographic resolution from matrix components. The former requirement was attained through the combined effects of 0.5% SDS and 30% acetontrile in the displacing reagent. Concentrations of acetonitrile greater than 30% are to be avoided due to resultant protein precipitation and compromise of the integrity of the ultrafiltration membrane. A 1:1 mixture of displacer and plasma results in greater than 97% recovery of temafloxacin and its metabolites.

Chromatography

All recently developed quinolone antibacterials are zwitterions in the pH range routinely used in reversed-phase CLC. Additionally, they are weak chelators of divalent cations. As a result, they typically chromatograph asymmetrically. This behavior is attenuated in mobile phases which have high ionic strength or high acidity, and which commonly contain modifiers such as tertiary amine salts, citric acid, or perchloric acid. Previously, Adsorbosphere HS C_{18} columns were found to be superior in the chromatography of quinolones [9]; however, some peak tailing has occasionally been observed with new columns, a characteristic which usually diminishes with use. It was discovered that incorporation of N-acetylhydroxamic acid into the mobile phase results in marked improvement in peak symmetry and column efficiency toward the quinolones. As a result, the reagent is routinely added to the mobile phase, regardless of the extent of column conditioning. The nature of the interaction is unknown, but probably relates to reduction of chelation and adventitious binding within the chromatographic system. SDS is also an integral component of the mobile phase, since it selectively causes increased retention of temafloxacin and its basic metabolites (EDA and MEDA) relative to plasma constituents which otherwise elute near the analytes.

At a flow-rate of 1.5 ml/min, temafloxacin should have a retention time of 6–9 min, with the internal standard (A-57084: a *p*-bromophenyl-substituted quinolone) eluting approximately 1.5 min later. Under these conditions, the trace level oxidative metabolites elute prior to temafloxacin. The combined levels of these metabolites are usually less than 1–2% of the corresponding levels of temafloxacin in urine or plasma; however, since they are eight to ten times more fluorescent

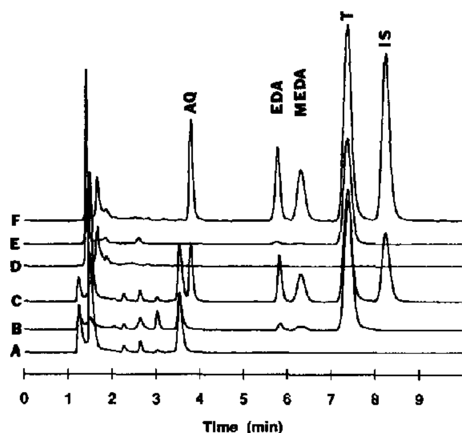

Fig. 2. Chromatograms of plasma ultrafiltrates and diluted urines. (A) Control plasma; (B) plasma from a subject receiving 200 mg temafloxacin; (C) plasma supplemented with 0.05 μg/ml each of metabolites AQ, EDA and MEDA, 1.0 μg/ml temafloxacin (T), and 0.5 μg/ml internal standard (IS); (D) 1:50 diluted control urine; (E) 1:200 diluted urine from a subject receiving 100 mg every 12 h; and (F) 1:50 diluted urine supplemented with temafloxacin, its metabolites and internal standard.

than temafloxacin, they are readily monitored. Sample chromatograms of processed urine and plasma samples are reproduced in Fig. 2. The relative retention times of temafloxacin and its metabolites are the following: (1) AQ, 0.3–0.4; (2) EDA, 0.70–0.75; (5) MEDA, 0.80–0.85; (6) temafloxacin, 1.00; and (7) internal standard, 1.10–1.15. Injection of 50 μl of a 5 ng/ml aqueous standard of temafloxacin typically produces a peak for temafloxacin that is two to three times higher than instrumental noise.

Precision and linearity

Plasma processing by ultrafiltration has been previously shown to be widely applicable for antimicrobial assays, providing very low intra-assay C.V.s: 2% for cefsolodin [11], 1% for cefmenoxime [12], and 0.5% for difloxacin [9]. Similar results were obtained with temafloxacin. The results of the evaluation of the

TABLE I

WITHIN-ASSAY PRECISION AND ACCURACY FOR THE DETERMINATION OF TEMAFLOXACIN AND ITS METABOLITES IN PLASMA

Supplemented samples were assayed in quadruplicate. Regression correlation coefficients (r) were determined with $1/C^2$ weighting, using results from all replicates.

Concentration (μg/ml)		Target (%)	C.V. (%)
Actual	Observed		
Temafloxacin (r = 1.000)			
0.050	0.051	101.0	0.74
0.100	0.098	98.0	0.78
0.200	0.198	99.0	1.04
0.500	0.493	98.6	0.39
1.00	1.00	99.7	0.56
2.00	2.01	100.5	0.71
5.00	5.04	100.7	0.65
10.00	10.26	102.6	0.80
Metabolite EDA (r = 0.999)			
0.010	0.010	97.5	5.10
0.050	0.048	96.5	3.50
0.200	0.197	98.6	2.00
0.500	0.508	101.5	2.80
1.000	1.030	103.0	1.80
Metabolite MEDA (r = 0.998)			
0.010	0.010	100.0	8.20
0.050	0.046	92.5	2.10
0.200	0.201	100.5	0.90
0.500	0.512	102.5	2.90
1.000	1.032	103.2	2.00

procedure in human plasma are outlined in Table I. Regression of the response versus concentration data revealed a highly linear relationship (r = 0.9998), without systematic bias or a statistically significant intercept. The mean intra-assay C.V. for the standards ranging from 0.05 to 10 μg/ml was 0.7 ± 0.2%. Within-assay C.V.s for the determination of metabolites EDA and MEDA averaged approximately 3%. The essentially constant C.V.s for temafloxacin over a 200-fold concentration range indicates that reciprocal squared concentrations ($1/C^2$) are the most appropriate approximation of reciprocal analytical variances as the weighting scheme for regression of calibration curves.

Inter-assay precision

Inter-assay variance is largely associated with errors in weighing, pipetting, and quantifying HPLC responses. In the first clinical study, inter-assay C.V.s for the determination of plasma temafloxacin concentrations were assessed from the results of analyses of quality control samples. After thirteen analyses spanning a 69-day period, interassay C.V.s of 1.98 and 1.88% were obtained for blinded samples supplemented with temafloxacin at 2.0 and 0.2 μg/ml, respectively. Regression correlation coefficients from the calibration curves averaged 0.9996. The procedure was subsequently transferred and revalidated at three contract laboratories, with comparable results. After analysis of over 12 000 samples from various clinical studies, inter-assay C.V.s, estimated from analyses of quality control samples, averaged 4% or lower for concentrations above 0.1 μg/ml. The interassay C.V. estimates for nine recent studies, involving 5300 samples and 80 analytical runs, are summarized in Table II.

The limit of quantitation was assigned as 0.01 μg/ml, and assay C.V.s for quality control samples supplemented with 0.015 μg/ml averaged under 9% across studies. Typical therapeutic regimens of temafloxacin are 400 and 600 mg twice daily, which produce steady-state maxima averaging around 4 and 6 μg/ml,

TABLE II

BETWEEN-ASSAY PRECISION FOR THE DETERMINATION OF TEMAFLOXACIN IN PLASMA SUMMARIZED FOR NINE RECENT STUDIES

Study-wide between-assay coefficients of variation for duplicate analyses of quality control (QC) samples from a total of 80 analytical runs are summarized across nine recent studies. Calibration curve regression correlation coefficients averaged over 0.999 in all studies.

	Between-assay coefficients of variation (%)		
	Low QC (0.015 μg/ml)	Medium QC (0.15–0.40 μg/ml)	High QC (1.5–2.4 μg/ml)
Mean	8.8	4.0	2.9
S.D.	3.5	1.7	0.9
Low	4.9	2.1	1.6
High	13.3	7.5	4.6

respectively; thus, the assay can monitor temafloxacin elimination through ten half-lives. If desired, quantitation limits below 1 ng/ml are attainable using the extraction procedure, since it allows concentration of the analytes from the sample.

The precision of the assay of temafloxacin in urine is comparable to that in plasma. Quality control samples supplemented with 3, 15, and 75 μg/ml temafloxacin were assayed in triplicate or quadruplicate in six runs, two of which employed the alkaline hydrolysis procedure (Table III). At the end of the analysis, inter-assay coefficients of variation ranged from 1.8 to 3.5%, and mean accuracies of the determinations ranged from 99.3 to 103.5%. The precision of the determination of metabolites EDA and MEDA was comparable to that for temafloxacin. Use of the assay in clinical studies has shown that the extent of conjugation of temafloxacin is usually very low, typically accounting for less than 5% of the dose. Metabolites EDA, MEDA, and AQ typically account for less than 1% of the dose, with EDA and especially AQ being excreted predominantly as conjugates.

Stability in plasma and urine

Chemically, temafloxacin is stable, showing minimal changes when heated in the dry state at 110°C for 30 days, or at reflux for 3 h in weak aqueous acid or

TABLE III

ASSAY PRECISION AND LINEARITY FOR THE DETERMINATION OF TEMAFLOXACIN AND ITS METABOLITES IN URINE

Regression correlation coefficients, mean concentrations and coefficients of variation are given for quality control samples assayed in quadruplicate or triplicate over six days for temafloxacin and over four days for the metabolites EDA and MEDA.

Concentration (μg/ml)		n	C.V. (%)
Actual	Observed (mean)		
Temafloxacin (r = 0.9992)			
75	77.66	22	2.7
15	14.89	22	3.5
3	3.03	22	1.8
Metabolite EDA (r = 0.9995)			
3.75	3.95	15	2.6
0.75	0.80	15	2.6
0.15	0.158	15	2.3
Metabolite MEDA (r = 0.9998)			
3.75	3.74	15	3.0
0.75	0.75	15	2.6
0.15	0.146	15	2.8

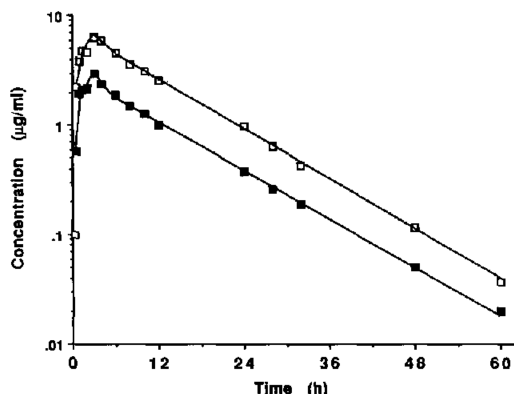

Fig. 3. Plasma temafloxacin concentration profiles of a subject receiving 400- and 1000-mg doses.

base. Since metabolism of temafloxacin is minimal, no degradation would be expected in frozen plasma or urine. Routinely, when fresh calibration curves are prepared, standards from the preceding curves are assayed as unknowns. Plasma and urine calibrators, stored frozen as long as 1.25 and 1.5 years, respectively, have been reassayed, typically with the finding that observed concentrations are within 7% of the nominal values. Although no degradation of metabolites EDA and AQ has been noted, 15% loss of metabolite MEDA was found in frozen samples averaging 1.1 years in age. As a further demonstration of stability, temafloxacin stored in plasma at room temperature for 25 days gave no indication of degradation, averaging 101.5 ± 3.3% of theory after eight analyses.

Clinical applications

The procedures described above have been employed throughout the clinical studies and in preclinical pharmacokinetic, metabolic, and toxicology studies. Interference in the determination of temafloxacin has not been found in the analyses of predosing samples from patients taking a variety of medications, including patients with renal failure. In addition to plasma and urine, the procedures have been extended to the analysis of bile, cerebrospinal fluid, sputum, and tissue homogenates. The normal column lifetime is between 2500 and 3000 samples. Owing to the simplicity of the work-up, analysis rates of 80–120 samples per day are routinely accomplished by one technician, using a single instrument equipped with an autoinjector. Typical results of the procedure are shown in Fig. 3 for a subject receiving single oral 400 and 1000-mg doses. The mean estimates of the terminal phase half-life and renal clearance for this subject were 7.9 h and 111 ml/min, respectively.

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