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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURES FOR THE DETERMINATION OF DIFLOXACIN AND ITS METABOLITES IN BIOLOGICAL MATRICES

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SUMMARY

A simple and extremely precise high-performance liquid chromatographic procedure has been developed for the determination of difloxacin and its metabolites in plasma and urine. Work-up of plasma samples entails ultrafiltration after addition of an internal standard in a displacing reagent containing sodium dodecyl sulfate. The ultrafiltrates are directly analyzed using a C_{18} reversed-phase analytical column, a soap-chromatographic mobile phase, and a fluorescence or ultraviolet detector. The mean intra-assay coefficient of variation for difloxacin over a concentration range of 10 ng/ml to 10 μg/ml was 0.5% when fluorescence detection and an internal standard were employed. Inter-assay coefficients of variation were approximately 2%. Recoveries of difloxacin and its metabolites were essentially quantitative and calibration curves were strictly rectilinear.

INTRODUCTION

Difloxacin is a synthetic quinolone antibacterial agent with high activity against gram-positive and gram-negative bacteria (Fig. 1). It is extensively metabolized [1,2], primarily by glucuronidation and by oxidation to the N-oxide and N-desmethyl analogues. Bioassays have been developed for difloxacin; however, they are considered inferior to high-performance liquid chromatographic (HPLC) procedures, particularly when precision, sensitivity, and specificity are of primary importance (e.g., pharmacokinetic or metabolic studies). The recent quinolone carboxylic acid antibacterials all possess strongly basic centers, usually a piperazinyl moiety; hence, they are ionized at all pH values. Consequently, non-ideal chromatographic interactions and low solubility in many organic solvents restrict the analytical options for this class of compounds. Typical analytical procedures for congeners of difloxacin involve either extraction with chloroform or methylene chloride [3–10] or deproteinization [11–13], generally followed by

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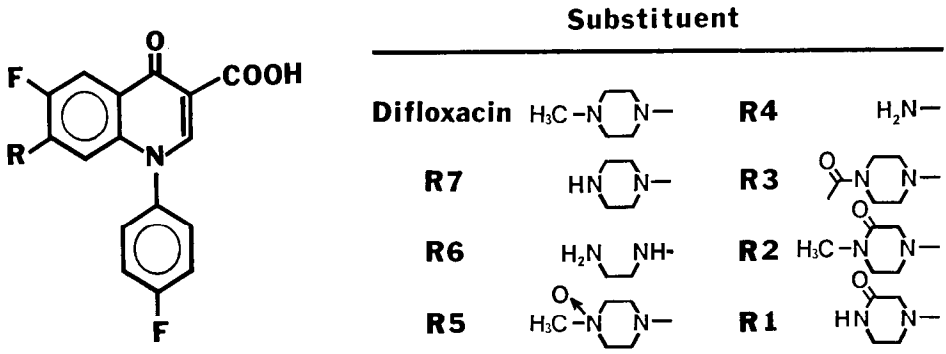

Fig. 1. Chemical structures of difloxacin and potential oxidative metabolites.

reversed-phase [6,7,9,11–15] or ion-exchange [4,10] chromatography. Described herein is an analogous extraction procedure, as well as an alternative procedure, with greatly improved precision and simplicity, for the determination of difloxacin and its metabolites.

EXPERIMENTAL

Chromatography

HPLC analyses were conducted using a Spectra-Physics (San Jose, CA, U.S.A.) Model 8100 chromatograph in conjunction with a Model FS970 Kratos (Ramsey, NJ, U.S.A.) spectrofluorimeter (389-nm emission filter) and an LDC (Riviera Beach, FL, U.S.A.) ultraviolet spectrophotometer, connected in tandem. Both detectors were operated at a wavelength of 280 nm. The C_{18}-Adsorbosphere-HS® analytical column (Alltech, Deerfield, IL, U.S.A.; 250×4.6 mm, 7-μm particles) was used at ambient temperature. The mobile phase, typically containing 0.05 *M* phosphate, 0.2% sodium dodecyl sulfate (SDS), and 50% HPLC-grade acetonitrile, was pumped at 1.5 ml/min. The fluorescence intensity of difloxacin and its metabolites decreases as the pH of the mobile phase is increased from 2.5 to 5.5. In this pH range, the relative retention of the metabolites is relatively constant, with the exception of the N-oxide, which is more retained as pH increases. Mobile phases of low pH (2.5–2.9) are normally employed in the analysis of plasma when maximum sensitivity is required; however, for the analysis of urine specimens, which are typically diluted 1:10 to 1:20, the mobile phase pH is adjusted to the 4.8–5.2 range. For urine or plasma, injection volumes range from 10 to 50 μl, depending on difloxacin dosage.

Reference standards of difloxacin and its metabolites were synthesized at Abbott Labs. Identities of the metabolites in biological specimens were established by chromatographic and spectral (UV/fluorescence response ratios) comparisons to the reference compounds, using several eluents. In addition, difloxacin glucuronide and the N-desmethyl and N-oxide metabolites were chromatographically isolated from biological specimens and were subjected to electron-impact (EI) and fast atom bombardment (FAB) mass spectral confirmation.

Ultrafiltration procedure

Plasma ultrafiltrates were prepared using the Amicon (Lexington, MA, U.S.A.) Centrifree™ micropartition system. The Centrifree apparatus is preferred over the Centriflo™ device for the following reasons: (1) its "YMT" membrane has a lower binding affinity toward difloxacin; (2) it is disposable; (3) its detachable receptacle is convenient for storage of the ultrafiltrates; and (4) it allows work-up of small volumes (100 μl of ultrafiltrate can be obtained from 150 μl of serum in 20 min).

Since difloxacin is 42% bound to plasma proteins and binds slightly to the Centrifree membrane, a displacing reagent is required to achieve quantitative recoveries. This is accomplished by addition of a mixture containing 0.5% SDS (Aldrich, Milwaukee, WI, U.S.A.) and 28% acetonitrile in 0.1 *M* phosphate, pH 7.1, to an equal volume of plasma. Higher concentrations of acetonitrile cause protein precipitation and compromise of the membrane integrity, and thus should be avoided. The displacing reagent normally contains 0.5–1.0 μg/ml of the *p*-methylphenyl analogue of difloxacin as the internal standard. This reagent (0.5 ml) is dispensed by a Micromedic pump (Philadelphia, PA, U.S.A.) into 10×75 mm tubes, into which the plasma samples (0.5 ml) are subsequently added. Assay precision depends more on the reproducibility of these first two steps than on any other factor. After transfer of the mixtures to Centrifree apparatus and 20 min centrifugation (1500 *g*), the ultrafiltrates are then injected without further treatment. With the use of an autoinjector, one analyst can process 120 samples per day.

Extraction procedure for plasma and urine

The ultrafiltration procedure is generally preferred due to its superior simplicity and precision; however, the extraction procedure offers advantages in terms of greater ultimate sensitivity (<1 ng/ml) and reduced background from matrix components. Essentially quantitative recoveries of difloxacin and its principal active metabolite, N-desmethyldifloxacin, can be obtained by a single extraction with five volumes of chloroform or methylene chloride. Typically, 0.5-ml aliquots of plasma or urine and 0.5-ml aliquots of internal standard (1 or 5 μg/ml for plasma or urine, respectively) in 0.5 *M* phosphate buffer (pH 7.2) are mixed and extracted 10 min by horizontal low-speed agitation (Eberbach, Ann Arbor, MI, U.S.A.) with 5 ml of HPLC/Spectro-grade methylene chloride (Alltech). After centrifugation (10 min at 900 *g*) and evaporation of the organic phase, the residue is reconstituted with mobile phase.

RESULTS AND DISCUSSION

Clean-up procedures

Procedures other than extraction and ultrafiltration were investigated, subsequently to be abandoned. Protein precipitation with acetonitrile and trichloroacetic acid gave unacceptable recoveries, presumably due to coprecipitation of the analytes with the plasma proteins. Disposable octadecyl columns gave nearly quantitative recoveries of difloxacin, with minimal plasma-related background

peaks; however, their use was disfavored due to low recoveries of the metabolites and flow-sensitive recoveries of difloxacin.

For drug level analyses that do not require a concentrative step, ultrafiltration procedures [16,17] are unrivaled in simplicity, and thus in precision. The two major obstacles in the use of the procedures are: (1) obtaining quantitative displacement of the analytes from the plasma proteins and (2) optimizing chromatographic conditions so that drug, metabolites of interest, and internal standard are resolved from matrix components. Difloxacin binds both to plasma proteins and to the membrane of the Centrifree ultrafiltration apparatus. Although SDS has been successfully employed as a displacing reagent for protein-bound antibiotics [17], recoveries greater than 90% could not be attained for difloxacin and its metabolites after addition of either SDS or sodium 1-octanesulfonate (OSA), at final concentrations up to 1.0%.

Quantitative recoveries were attained with 0.7% SDS in 0.07 *M* sodium hydroxide as the displacer; however, the glucuronide conjugates of difloxacin, and its metabolites, which are collectively present in plasma at concentrations up to 7% of those of parent drug, are slowly hydrolyzed in the alkaline ultrafiltrates. This minor problem is obviated either by neutralization or by the use of 0.5% SDS and 28% acetonitrile in phosphate buffer (pH 7) as the displacing reagent.

The extraction procedure described in the Experimental section offers several advantages over the ultrafiltration procedure: (1) lower assay costs; (2) extension of the limit of quantification to below 1 ng/ml; and (3) reduced background from matrix components. There are, however, attendant disadvantages: (1) precision is reduced; (2) recoveries of the polar metabolites are reduced; (3) more manipulations are required; and (4) work-up time is trebled. Nonetheless, for samples with abnormal matrix interference, extraction may be the procedure of choice.

Chromatography

With many of the commercially available reversed-phase chromatographic columns investigated, the quinolones chromatographed as severely tailing peaks. This behavior was reduced, but not eliminated, in mobile phases with high acidity or ionic strength. Buffers containing citric acid, perchloric acid or tertiary amine salts also generally improved peak symmetry; however, C_{18}-Adsorbosphere-HS provided superior chromatograms in phosphate-based mobile phases.

From analogy to the metabolism of other quinolones [3,4,6,7,10,11] seven reference compounds were synthesized as potential metabolites in various animals and man (Fig. 1). Without the addition of ion-pairing reagents to the mobile phase, resolution of the metabolites of difloxacin from several troublesome plasma constituents could not be achieved by manipulation of the pH, buffer ionic strength or organic modifier of the mobile phase. Addition of tetrahydrofuran to acetonitrile-based mobile phases diminished the resolution between difloxacin and its N-desmethyl metabolite, whereas modification with methanol caused band-broadening. Attempts to selectively increase the retention of the analytes using octane sulfonic acid as an ion-pairing reagent were unsuccessful; however, with "soap" chromatography, employing 0.2% SDS, difloxacin and its metabolites are

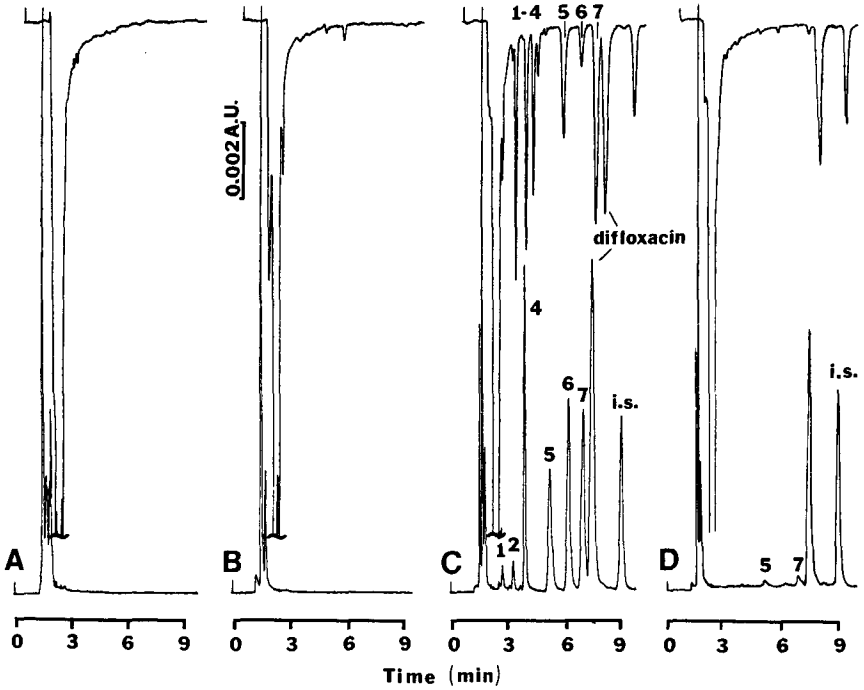

Fig. 2. Fluorescence (lower) and ultraviolet (upper) chromatograms of (A) 1:20 diluted control urine, and ultrafiltrates of (B) control plasma, (C) plasma supplemented with difloxacin, reference metabolites, and internal standard, and (D) plasma obtained 24 h after dosing from a subject receiving 200 mg difloxacin. HPLC conditions are described in the text; mobile phase pH was 4.9. More than 1700 plasma ultrafiltrates and urine specimens had been injected onto this column prior to these chromatograms. Peaks: 1-7 = metabolites R1-R7; i.s. = internal standard.

more retained, eluting in a region of the chromatogram of normal plasma and urine (from humans, dogs, rats, mice, rabbits, and monkeys) that is essentially devoid of endogenous components (Fig. 2).

Detection of difloxacin and its metabolites

The molar extinction coefficients at 280 nm for difloxacin and its metabolites are approximately $4 \cdot 10^4$; hence, under routine chromatographic conditions, quantities in the order of 1 ng can be detected. The absorption spectra also have secondary maxima in the range of 310–330 nm with extinction coefficients of approximately $1.5 \cdot 10^4$.

The relative fluorescence efficiency (RFE) of the metabolites, when compared to difloxacin (RFE = 1.0), varies considerably. In mobile phases at pH 5, using an excitation wavelength of 280 nm, metabolites R7 (RFE = 0.5), R6 (RFE = 2.7), R5 (RFE = 0.5) and R4 (RFE = 4.1) are easily detected fluorimetrically; however, metabolites R1, R2 and R3 have low intrinsic fluorescence (RFE < 0.07) and require adjunctive ultraviolet detection (Fig. 2). When maximum sensitivity is required, more acidic mobile phases (pH 2.5–3) are employed, allowing detection of 0.1 ng of difloxacin.

TABLE I

PRECISION AND RECOVERY OF THE ULTRAFILTRATION PROCEDURE FOR DIFLOXACIN

The 0.02, 0.2 and 2.0 standards were assayed in quintuplicate; others were triplicate. Weights for the linear regression of the results were reciprocal variances.

Concentration (μg/ml)	Coefficient of variation (%)		Regression deviation (%)		Recovery (%)
	Ultraviolet	Fluorescence	Ultraviolet	Fluorescence	
0.010	—	0.50	—	0.0	101.4
0.020	—	1.40	—	−0.5	100.0
0.050	5.3	1.03	−5.5	−1.2	99.8
0.100	3.3	0.33	−0.4	1.3	101.9
0.200	1.8	0.61	1.5	0.2	100.4
0.500	1.6	0.33	1.4	0.3	101.0
1.000	0.7	0.19	0.9	−0.5	101.6
2.000	1.7	0.36	2.6	0.5	101.7
5.000	0.3	0.33	0.0	−0.4	102.4
10.000	1.4	0.19	−6.8	0.0	101.0
Mean	2.0	0.53	2.4	0.5	101.1

Assay precision, linearity, and recovery

Over a difloxacin concentration range in plasma of 10 ng/ml to 20 μg/ml, the methylene chloride extraction procedure produced recoveries exceeding 97%, with a mean intra-assay coefficient of variation (C.V.) of 3%. Regression correlation coefficients typically exceeded 0.9995.

The precision and recovery characteristics of the ultrafiltration procedure are given in Table I. With the use of an internal standard and fluorescence detection, the mean assay C.V. for difloxacin standards ranging from 10 ng/ml to 10 μg/ml was 0.53%. With ultraviolet detection, background and plasma constituent noise increased the limit of quantification to approximately 0.05 μg/ml, and increased assay variance, particularly at low concentrations; nonetheless, the overall C.V. averaged only 2%. The quantitative recovery of difloxacin in the ultrafiltrates was invariant over a 1000-fold concentration range; hence, regression analysis revealed high linearity ($r=0.99999$) with minimal residual errors (mean absolute deviation = 0.5%). Based on the concentration-independent C.V.s with fluorescence detection, reciprocal variances are best approximated in the regression analyses of calibration curves by a reciprocal-squared concentration weighting scheme.

The inter-assay C.V.s for difloxacin and N-desmethyl difloxacin, assessed from quality-control samples used in the routine analysis of clinical samples, averaged 2.2 and 1.8%, respectively. The stability of these compounds in frozen plasma was assessed by repeated analyses over a two-month period. By linear regression, the slopes of the concentration versus time data over that period were not significantly ($p<0.05$) different from zero.

Ancillary experiments with standards of the oxidative metabolites have dem-

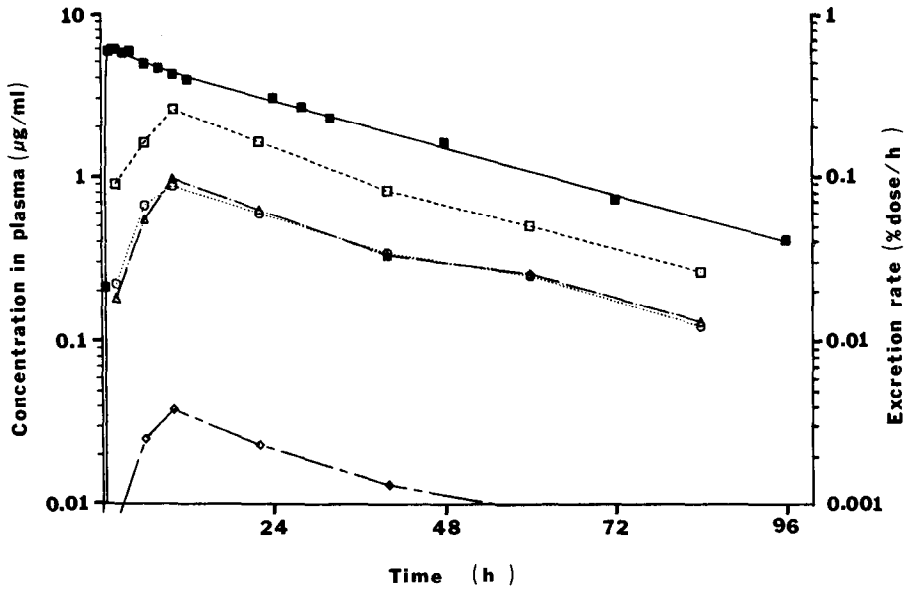

Fig. 3. Plasma level profile of difloxacin (■) and urinary excretion rate plots of difloxacin (□) and metabolites R7 (○), R5 (△), and R6 (◇) in a subject receiving a 600-mg oral dose.

onstrated that the ultrafiltration procedure produces recoveries exceeding 98%, with intra-assay C.V.s below 1%.

Clinical applications

In all species investigated, difloxacin is extensively metabolized; yet, in human plasma, the levels of the major oxidative metabolite N-desmethyldifloxacin (R7), are typically less than 5% of those of parent drug, primarily as a result of differences in renal clearance (Cl_r). Difloxacin is renally reabsorbed ($Cl_r < 10$ ml/min), whereas N-desmethyldifloxacin is both filtered and secreted ($Cl_r > 280$ ml/min). Typical results of the analytical procedure for plasma are shown in Fig. 3 for a subject receiving a 600-mg oral dose of difloxacin. Also presented are the urinary excretion rate plots for difloxacin and its major oxidative metabolites, R5, R6, and R7. The curves for parent drug and the unconjugated metabolites decline in parallel, with a terminal phase half-life of approximately 26 h. Low levels of a conjugate of metabolite R4 have also been observed in man; however, the presence of metabolites R1–R3 has not been definitively demonstrated. Total urinary recovery of difloxacin and its metabolites account for one third of the dose, the remainder being excreted by hepatobiliary processes.

The high recovery, precision, and simplicity of the ultrafiltration procedure is not unique to difloxacin. With proper selection of the displacing reagent and chromatographic conditions, it is widely applicable to other analytes for which a concentration step is not required. Unlike extraction procedures which are more labor-intensive and which usually fail to quantitatively recover polar metabolites, ultrafiltration is ideally suited for characterization of metabolic profiles in plasma.

Unlike other deproteinizing procedures, harsh reagents or substantial sample dilutions are avoided.

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