Journal of Chromatography, 530 (1990) 407–417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5378

Determination of amifloxacin and two of its principal metabolites in plasma and urine by high-performance liquid chromatography using automated column switching

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(First received January 9th, 1990; revised manuscript received April 23rd, 1990)

ABSTRACT

The automated determination of amifloxacin and two of its principal metabolites in human plasma and urine by column-switching high-performance liquid chromatography is described. Plasma or urine samples, diluted 1:1 with 0.5 *M* sodium citrate buffer pH 2.5, were directly injected onto a cation-exchange pre-column. Following a 2.0-min wash of the pre-column with water at a flow-rate of 1.1 ml/min, the effluent from the pre-column was directed to the analytical column by a column-switching device. The precision of the plasma and urine methods ranged from a ±1.9 to ±3.6% for all compounds. The accuracies of the methods were within a range of −3.3% to 6.4% of the nominal values for all compounds. Linear responses were observed for all the standards in the range 0.10–5.0 μg/ml for plasma and 0.50–100 μg/ml for urine for all three compounds. The minimum quantifiable levels were 0.10 and 0.50 μg/ml for plasma and urine, respectively. The analytical methods may be used to quantify amifloxacin and the piperazinyl-N-desmethyl and piperazinyl-N-oxide metabolites in plasma and urine samples obtained from humans, monkeys, dogs and rats.

INTRODUCTION

Amifloxacin (formerly Win 49375), 6-fluoro-1,4-dihydro-1-(methylamino)-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (Fig. 1), is a fluoroquinolone carboxylic acid with demonstrated potency against a broad spectrum of gram-negative organisms and potential for treating some gram-positive infections [1–3]. It is stable to β-lactamase and aminoglycoside-modifying enzymes and could, therefore, provide an alternative to the currently used penicillins, cephalosporins or aminoglycosides [4].

A previous method based on high-performance liquid chromatography (HPLC) for the determination of amifloxacin and the piperazinyl-N-desmethyl metabolite involved a tedious and time-consuming double extraction procedure and did not allow extraction of the piperazinyl-N-oxide metabolite [5]. Therefore, a new automated method which would reduce the time for sample preparation and permit quantification of the piperazinyl-N-oxide metabolite was developed. This report describes a method based on HPLC using column switching [6,7] for

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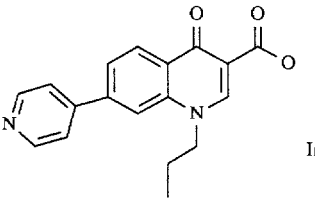
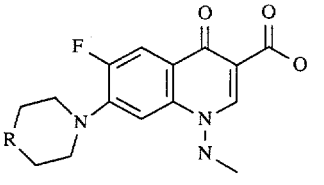


Internal Standard



Compound	R	Mol. Wt.
Amifloxacin	N-CH_3	334.4
N-desmethyl	N-H	320.3
N-oxide	CH_3-N^+-O^-	350.3

Fig. 1. Structures of amifloxacin, its principal metabolites and the internal standard.

the determination of plasma and urine concentrations of amifloxacin, the piperazinyl-N-desmethyl metabolite (N-desmethyl), and the piperazinyl-N-oxide metabolite (N-oxide).

EXPERIMENTAL

Reagents

Amifloxacin, two of its principal metabolites [8] and the internal standard were synthesized at Sterling Research Group and used without further purification. Acetonitrile was of the HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was redistilled and purified with a Barnstead Organipure™ (Boston, MA, U.S.A.) system. All other chemicals were obtained commercially (reagent grade or better) and used without further purification.

Standard solutions

Stock standard solutions were prepared by dissolving amifloxacin, N-desmethyl and N-oxide in 0.01 *M* sodium hydroxide to achieve final concentrations of each analyte to 100 μg/ml for plasma standards and 1000 μg/ml for urine standards. These stocks were then diluted to achieve stocks from which a 50-μl aliquot was used to prepare calibration curve standards for plasma and 100-μl aliquot for urine calibration curve standards.

Preparation of calibration curve standards

Appropriate aliquots of standard solutions were pippeted into 1.0 ml of either control human plasma (containing potassium oxalate anticoagulant) or control human urine to give calibration curve standard concentrations of 0 (0.01 *M* sodium hydroxide compensate), 0.01, 0.25, 0.50, 0.75, 1.0, 2.5 and 5.0 μg/ml in plasma and 0 (0.01 *M* sodium hydroxide compensate), 0.50, 1.0, 5.0, 10, 25, 50, 75 and 100 μg/ml in urine.

Preparation of spiked plasma and urine samples

Sets of randomized spiked samples were prepared for analysis under single-blind conditions for plasma and urine. The plasma set contained triplicate samples at final concentrations of 0 (0.01 *M* sodium hydroxide compensate), 0.13, 0.52, 0.96 and 3.5 μg/ml. The urine set contained triplicated samples of 0 (0.01 *M* sodium hydroxide compensate), 0.57, 5.7, 43 and 84 μg/ml. Samples were analyzed immediately after preparation. (A previous report indicated that freezing the samples (six days for plasma, ten days for urine) did hot have any appreciable influence upon assay values for amifloxacin [5]. Data from our laboratory confirm these findings for amifloxacin and also indicate metabolite concentrations are not appreciably affected when frozen for similar lengths of time.)

Sample preparation procedure

To each plasma calibration curve standard or spiked sample (1.0 ml) were added 100 μl of the internal standard solution (20 μg/ml in 0.01 *M* sodium hydroxide). To each urine calibration curve standard or spiked sample (1.0 ml) were added 100 μl of the internal standard solution (400 μg/ml in 0.01 *M* sodium hydroxide). A 1-ml volume of 0.05 *M* sodium citrate buffer, pH 2.5, was then added, followed by vortex-mixing for approximately 15 s. Centrifugation at approximately 950 *g* for 7–10 min was performed to remove any precipitation. An appropriate aliquot (100 μl for plasma, 50 μl for urine) of supernatant was then injected onto the chromatographic system.

Instrumentation and chromatography conditions

The HPLC system (Fig. 2) included two columns: a customized ion-exchange column (Bondapak™ CX Corasil, 37–50 μm particle size, 5 cm × 2 mm I.D.; Waters Assoc., Milford, MA, U.S.A) was employed for sample clean-up and

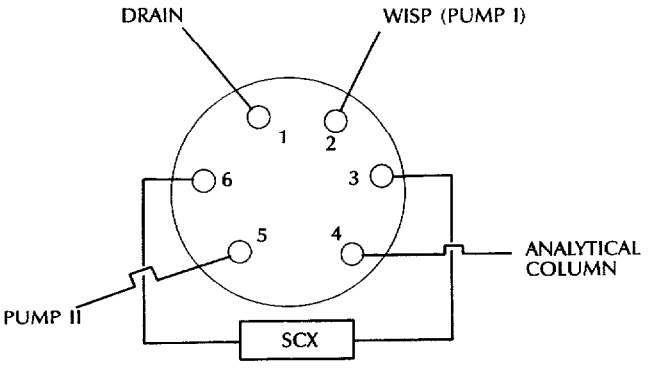


Fig. 2. Schematic of the switching valve interfaced to the chromatographic system.

concentration, and a chemically bonded phase of the C_{18} type (reversed phase) was used for analytical separation (Nova-Pak C_{18}, 4-μm Radial-Pak cartridge in a Z-module, Waters Assoc.). The pre-column for the analytical column consisted of Bondapak C_{18} Corasil (37–50 μm particle size, 23 mm × 3.9 mm I.D.; Waters Assoc.).

Samples were injected onto the ion-exchange column and washed with distilled water (1.1 ml/min) for 2 min. Column eluent passed through a switching valve (Rheodyne 7000, Rheodyne, Cotati, CA, U.S.A.). Concurrently, the analytical mobile phase (0.3 *M* monobasic potassium phosphate adjusted to pH 2.2 with orthophosphoric acid–acetonitrile, 87.5:12.5, v/v) was directed through the analytical column at a flow-rate of 2.0 ml/min. At 2 min, the switching valve redirected the flow of analytical mobile phase such that it passed through the ion-exchange column in a backflush mode eluting the sample onto the analytical column. This was continued for 3 min at which time the switching valve completed its cycle and the ion-exchange column was re-equilibrated with distilled water in preparation for the injection of the next sample. This process was automated using a Hewlett Packard sampler-event control module interfaced with an HP3357 LAS computer system (Hewlett Packard, Palo Alto, CA, U.S.A.). The system was operated at room temperature.

The analytical system also included two solvent-delivery pumps (Waters M-45, Waters Assoc. and Milton Roy mini pump, Riviera Beach, FL, U.S.A.), a Waters Model 440 UV detector with a 280-nm filter (Waters Assoc.) and a WISP sample processor (Waters Assoc.).

Data processing

The output from the detector was interfaced with a Model 3357 laboratory automation system (Hewlett Packard) for data acquisition and handling. Drug concentrations were determined by inverse prediction from a linear regression of standard peak-height ratios as a function of standard concentrations [9].

The observed concentrations for the prepared, spiked samples were expressed as percentage difference from nominal values, and the range of these percentages was used to define the accuracy of the assay. Precision was estimated from the standard deviation derived from the analysis of variance on the percentage differences.

Recovery

In a separate experiment, two sets of samples were prepared for both plasma and urine; one set was diluted with 0.25 *M* citrate buffer pH 2.5 (2.0 ml) and the second set was prepared in the appropriate biological medium. Each set consisted of triplicate samples prepared at each of three concentrations (0, 0.25, 1.0 and 2.5 μg/ml for plasma; 0, 5, 25 and 75 μg/ml for urine). The set diluted with only citrate buffer was injected directly onto the analytical column and the mean peak area was compared to that obtained from samples prepared in the biological medium and injected onto the two-column system. The overall percentage recovery was based upon the mean value obtained from all concentrations.

A similar procedure was employed to determine the recovery of the internal standard; however, recoveries were estimated only at 1.0 μg/ml in plasma ($n = 12$) and 20 μg/ml in urine ($n = 12$).

RESULTS AND DISCUSSION

Chromatography

Fig. 3 shows representative chromatograms of processed plasma samples. Fig. 3A shows a processed control human blank. The chromatogram of this blank sample indicates that there are no apparent interferences from endogenous compounds. Fig. 3B shows a processed control human plasma spiked with internal standard and 0.5 μg/ml each of amifloxacin, N-desmethyl and N-oxide. Fig 3C shows a plasma sample from a healthy volunteer taken just prior to an 18 mg base per kg infusion of amifloxacin mesylate after two days of q12h (every 12 h) administration. Approximate retention times were 6.9 min for N-desmethyl, 7.7 min for amifloxacin, 8.8 min for N-oxide and 10.2 min for the internal standard. All peaks of interest were well separated. Fig. 4 shows representative chromatograms of processed human urine samples. Fig. 4A shows a processed control human urine blank and, again, indicates that there are no apparent interferences from endogenous compounds. Fig. 4B shows a processed control human urine spiked with internal standard and 10 μg/ml each of amifloxacin, N-desmethyl and N-oxide. Fig. 4C shows a urine sample from a healthy volunteer taken just prior to a 12 mg base per kg infusion of amifloxacin mesylate after two days of q8h administration. Retention times for compounds of interest were equivalent to those reported for analysis of plasma, and all peaks of interest were well separated.

Work prior to the validation of this method indicated that the chemically

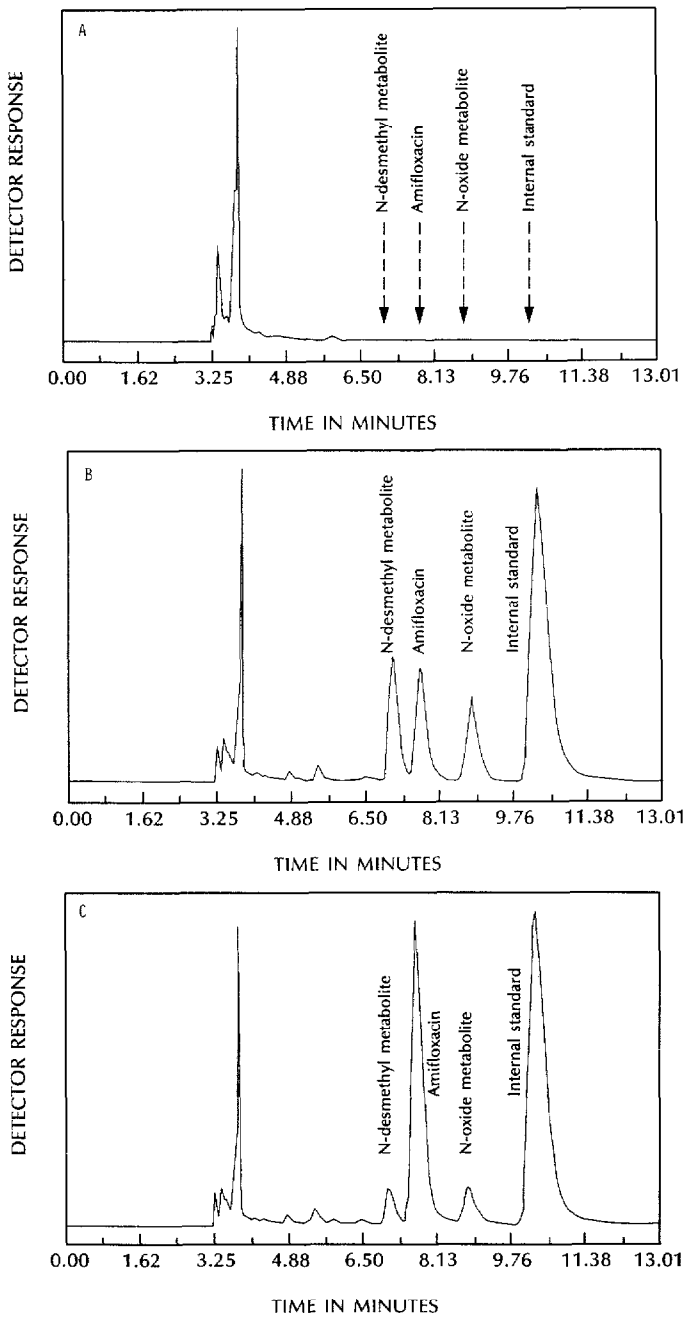


Fig. 3. Computer-generated chromatograms of plasma analysis. (A) Processed control human blank. (B) Processed control human plasma spiked with internal standard and 0.5 μg/ml each of amifloxacin and the N-desmethyl and N-oxide metabolites. (C) Plasma sample from a healthy volunteer taken just prior to an 18 mg base per kg infusion of amifloxacin mesylate after two days of q12h administration.

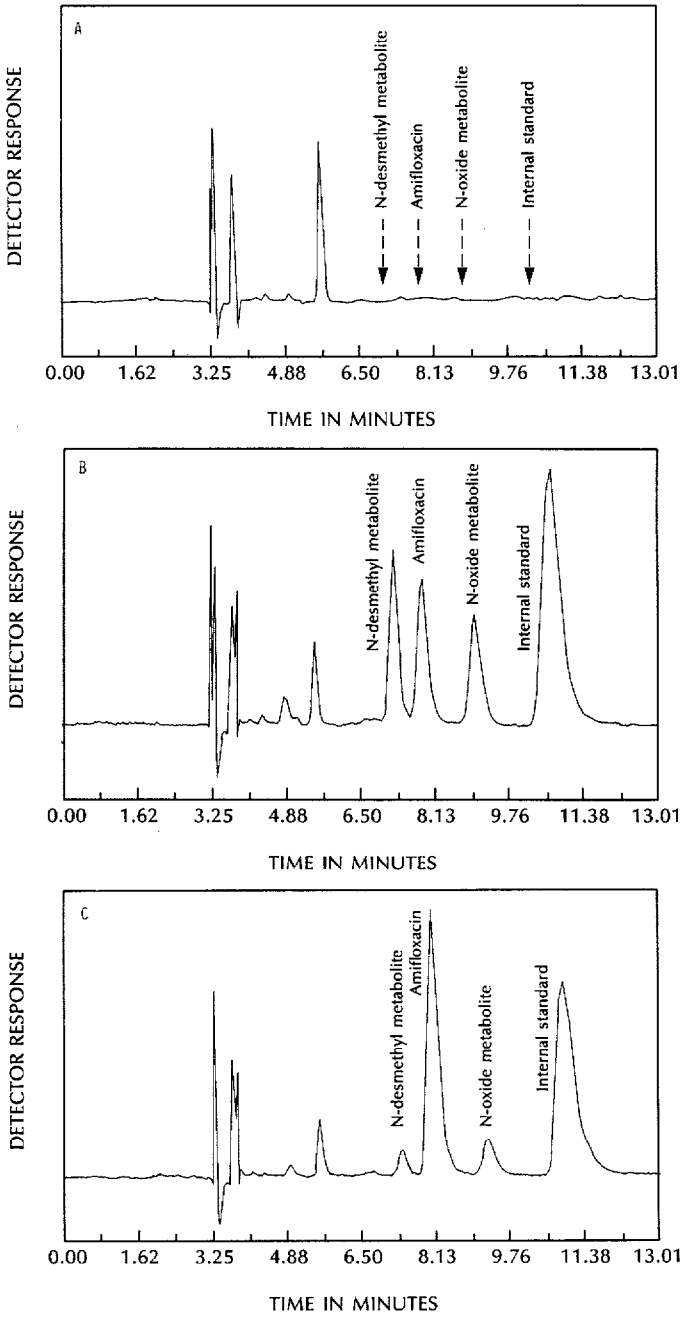


Fig. 4. Computer-generated chromatograms of urine analysis. (A) Proccessed control human blank. (B) Processed control human urine spiked with internal standard and 10 μg/ml each of amifloxacin and the N-desmethyl and N-oxide metabolites. (C) Urine sample from a healthy volunteer collected 6–8 h after a 12 mg base per kg infusion of amifloxacin after two days of q8h administration.

TABLE I

TYPICAL LINEAR REGRESSION PARAMETERS OF PEAK-HEIGHT RATIOS (DRUG OR METABOLITE/INTERNAL STANDARD) *VERSUS* CONCENTRATION

Concentration range (μg/ml)	Compound	Slope	Intercept (μg/ml)	Coefficient of determination (r^2)
Plasma				
0–5	Amifloxacin	0.8077	−0.0052	0.9999
	N-Desmethyl	0.9903	0.0036	0.9999
	N-Oxide	0.6259	−0.2251	0.9998
Urine				
0–100	Amifloxacin	0.0379	0.0120	0.9999
	N-Desmethyl	0.0427	0.0140	0.9999
	N-Oxide	0.0294	0.0070	0.9999

bonded phase of the C_{18} type produced higher resolution with an acceptable run time than others examined (*e.g.* Phenyl). Raising the pH of the analytical mobile phase reduced resolution where increasing ionic strength showed a slight improvement in resolution. Raising ionic strength also shortened retention times. However, it was felt that high ionic strengths decreased column life, therefore the mobile phase described in this method was chosen.

TABLE II

PRECISION, ACCURACY AND RECOVERY FOR THE ANALYSIS OF AMIFLOXACIN AND ITS METABOLITES IN PLASMA AND URINE

Compound	Precision[a] (%)	Accuracy[a] (%)	Recovery[b] (%)
Plasma			
Amifloxacin	±2.1	0.17 to 1.5	107
N-Desmethyl	±3.6	−3.3 to −0.19	102
N-Oxide	±3.2	0.33 to 6.4	104
Internal standard	±3.2		102
Urine			
Amifloxacin	±2.1	−2.9 to 0.99	102
N-Desmethyl	±2.5	−3.3 to 1.1	101
N-Oxide	±1.9	−3.0 to 0.73	102
Internal standard			102

[a] Concentrations ranged from 0.13 to 3.5 μg/ml for plasma and 0.57 to 84 μg/ml for urine; $n = 12$ for all compounds.

[b] Concentrations of amifloxacin and its metabolites ranged from 0.25 to 2.5 μg/ml for plasma and 5.0 to 75 μg/ml for urine; a concentration of 1.0 μg/ml for plasma and 20 μg/ml for urine was used for the internal strandard; $n = 12$ for internal standard; $n = 9$ for all other compounds.

Linearity and limit of detection

A regression analysis of the peak-height ratio *versus* concentration showed linearity over the range 0.1–5.0 μg/ml for plasma and 0.5–100 μg/ml for urine. The coefficient of determination was 0.999 or greater for every standard curve (Table I). The minimum quantifiable level was determined as the lowest standard which gave a regression-estimated concentration within 15% of the nominal concentration and was 0.1 μg/ml in plasma and 0.5 μg/ml in urine for all three compounds.

Precision and accuracy

A summary of the precision and accuracy of the methods is presented in Table II. Precision was defined as the overall standard deviation of the percentage differences from nominal and was ±3.6% or less for all compounds. The accuracy was defined as the overall range of mean percentage differences from nominal and was −3.3% to 6.4% or better for all compounds.

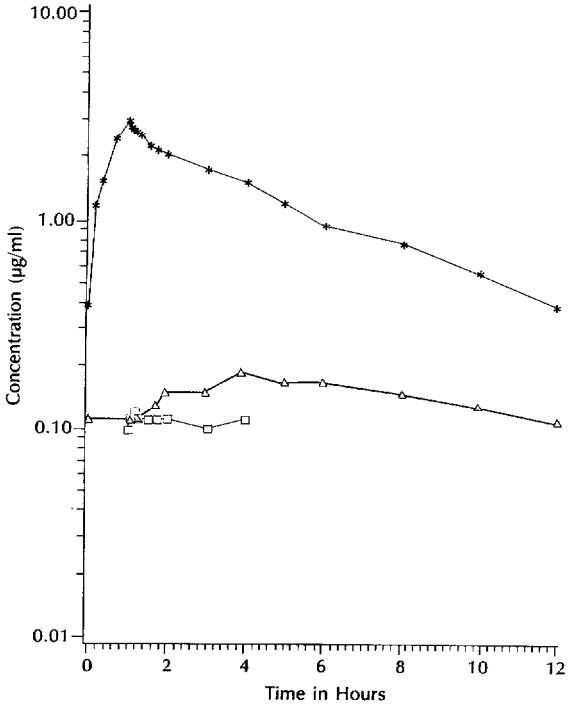


Fig. 5. Time course of plasma concentrations for amifloxacin (*) and the N-desmethyl (□) and N-oxide (△) metabolites in a healthy male volunteer following intravenous infusion of 3 mg base/kg amifloxacin mesylate after two days of q12h administration.

Recovery

The extraction efficiency was quantitative for all compounds in both assays. A summary of the recoveries is presented in Table II.

Utility

The utility of the method was demonstrated by the analysis of plasma and urine samples from rats, dogs, monkeys and humans from various studies. The time course of plasma concentrations for amifloxacin, N-desmethyl and N-oxide in a healthy volunteer following an intravenous dose of 3 mg base per kg during the third day of q12h administration of amifloxacin mesylate is presented in Fig. 5. A urine excretion rate plot for the same volunteer and day is presented in Fig. 6. This method has proven useful for analysis of specimens obtained from both clinical and animal studies.

The performance characteristics of the analytical methods were investigated over a five-month period. The mean (±S.D.) concentration for the frozen quality control samples of amifloxacin were 0.80 ±0.02 and 19.9 ±0.3 μg/ml for plasma (nominal concentration, 0.8 μg/ml) and urine (nominal concentration, 20.0 μg/ml), respectively, indicating no apparent degradation. Similarly, no apparent degradation was observed for N-desmethyl (0.80±0.4 and 20.0±0.5 μg/ml) or N-

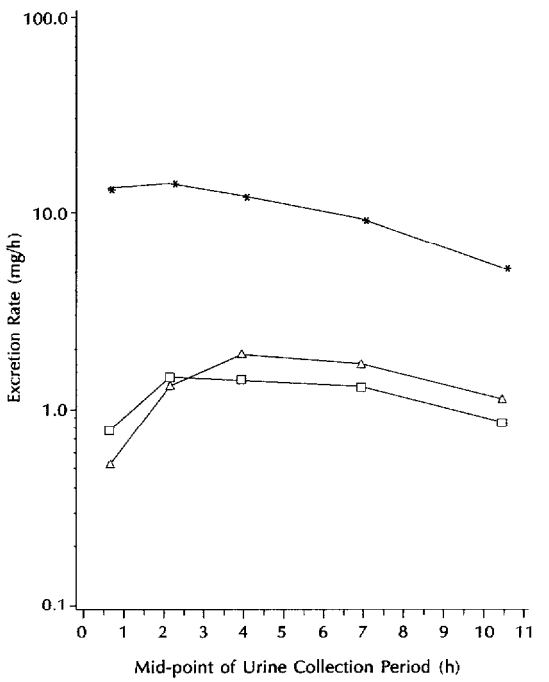


Fig. 6. Urine excretion rates for amifloxacin (*) and the N-desmethyl (□) and N-oxide (△) metabolites in a healthy volunteer following intravenous infusion of 3 mg base/kg amifloxacin mesylate.

oxide (0.81 ± 0.5 and 20.1 ± 0.7 μg/ml) in either frozen plasma or urine, respectively. The coefficient of determination (r^2) from each calibration curve was 0.998 or greater. The coefficients of variation for the mean values of the calibration curve slopes were 5% for the plasma analyses and 6% for the urine analyses (42 analytical runs for plasma, 19 analytical runs for urine). The ion-exchange column has performed satisfactorily for approximately 300–400 injections before routine replacement would occur.

CONCLUSION

A selective, accurate, reproducible, precise and automated HPLC assay has been developed for the determination of amifloxacin and two of its principal metabolites in plasma and urine. The tedious extraction step reported in a previous method has been eliminated. This method allows for shorter sample preparation time, which has proven helpful for analyzing the large number of samples required for pharmacokinetic studies.

ACKNOWLEDGEMENTS

We would like to acknowledge the assistance of Mr. Eric Gower in the preparation of this manuscript, Ms. Julie Utter for her technical assistance and Ms. Jackie Lang for the typing of this manuscript.

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