Pharmacokinetics of Ro 23-9424, a Dual-Action Cephalosporin, in Animals

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Ro 23-9424 is a dual-action cephalosporin with an aminothiazolylmethoxyimino-type side chain at the 7 position and feroxacin esterified at the 3' position. The new compound has broad and potent antibacterial activity in vitro and in vivo, reflecting contributions from both the β -lactam moiety and the quinolone moiety. In animals, the ester bond potentially could be hydrolyzed enzymatically or nonenzymatically, to yield the active metabolites desacetylcefotaxime and feroxacin. The extent to which Ro 23-9424 acts in vivo as a true dual-action cephalosporin, or acts as a combination of active metabolites, is therefore a function of its pharmacokinetic properties. To investigate these properties, Ro 23-9424 was administered as a single intravenous dose of 20 mg/kg of body weight to mice, rats, dogs, and baboons. Timed plasma samples were assayed by an ion-paired high-pressure liquid chromatography method that allowed detection of both intact Ro 23-9424 and feroxacin. The pharmacokinetic parameters of Ro 23-9424 were similar to published results for cefotaxime, while concentrations of feroxacin in plasma were low and fairly constant (about 1 to 3 μ g/ml) in all species, suggesting that excretion of the intact molecule is a major route of elimination for Ro 23-9424, as it is for cefotaxime. For technical reasons, urinary recovery of Ro 23-9424 was not quantitated, but intact Ro 23-9424 was found in high concentrations (>400 μ g/ml) in mouse urine aspirated directly from the bladder. In all species, low concentrations of free feroxacin in plasma persisted after the elimination of Ro 23-9424 was complete, but feroxacin did not accumulate unduly in a 14-day multiple-dose experiment in baboons. Thus, it seems likely that the activity seen in vivo is primarily due to intact Ro 23-9424, although the low levels of free feroxacin may also have some therapeutic significance.

Ro 23-9424 is a cephalosporin with an aminothiazolylmethoxyimino-type side chain at the 7 position and feroxacin esterified at the 3' position (1). Ro 23-9424 has broad and potent antibacterial activity in vitro (2, 9, 12) and in vivo (3). The antibacterial spectrum reflects contributions of both the cephalosporin moiety (notably, streptococci) and the quinolone moiety (notably, β -lactamase-overproducing strains). Ro 23-9424 binds to penicillin-binding proteins and inhibits replicative DNA biosynthesis (8). These observations have led to the use of the term "dual-action cephalosporins" for Ro 23-9424 and analogous compounds (1, 8).

It was originally proposed by O'Callaghan et al. (15) that the second "warhead" of such a cephalosporin, in this case, the feroxacin moiety of Ro 23-9424, is released when the β -lactam ring is opened by a bacterial enzyme, such as a β -lactamase. In the case of Ro 23-9424, active quinolone may also be released by enzymatic or nonenzymatic hydrolysis of the ester linkage. It is well known that 3'-acetoxy cephalosporins, such as cephalothin, cephapirin, and cefotaxime, are deesterified in vivo to form 3-hydroxymethylcephalosporins (4, 5, 19). These metabolites have antibacterial activity, but they are less potent than the acetylated cephalosporins. Hydrolysis of the ester bond of Ro 23-9424 presumably results in the formation of feroxacin and the 3-hydroxymethylcephalosporin desacetylcefotaxime, both of which have antibacterial activity. The extent to which Ro 23-9424 exerts the proposed mechanism of action in vivo (wherein the quinolone is released by a bacterial process), or acts as a combination of active metabolites, is therefore determined by its pharmacokinetic properties. This study

was designed to investigate the pharmacokinetics of Ro 23-9424 in four species of animals.

(A preliminary account of this work has been presented previously [J. G. Christenson, K. K. Chan, H. H. Farrish, I. H. Patel, and A. Specian, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 449, 1988].)

MATERIALS AND METHODS

Materials. Ro 23-9424 was synthesized in the Department of Anti-Infective Chemistry, Hoffmann-La Roche Inc., Nutley, N.J., and was used as the dihydrochloride salt, Ro 23-9424/002, or the monohydrochloride salt, Ro 23-9424/003. Hexadecyltrimethylammonium bromide was from Sigma Chemical Co. (St. Louis, Mo.). Acetonitrile and water for high-pressure liquid chromatography were high-pressure liquid chromatography grade from Fisher Scientific Co., Pittsburgh, Pa.

Serum protein binding. Protein binding in serum of various species was determined by using a centrifugal ultrafiltration method (Centrifree Micropartition System; Amicon Corp., Lexington, Mass.) after incubation at 37°C for 10 min. Freshly collected serum samples were spiked to contain concentrations of Ro 23-9424 from 3 to 90 μ g/ml.

Pharmacokinetic studies. For single-dose studies, Ro 23-9424 was administered intravenously to mice, rats, dogs, and baboons at 20 mg/kg of body weight. Blood samples were obtained at appropriate intervals using heparin (Eli Lilly & Co., Indianapolis, Ind.) as anticoagulant. Plasma was separated immediately, and an equal volume of acetonitrile was added. Precipitated protein was removed by centrifugation, and the supernatants were stored at -70°C . There was no

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apparent degradation of samples that were treated and stored in this manner for up to 2 years.

In mice (CD₁ Swiss Albino; weight, 18 to 20 g each), drug administration was via the tail vein. Four mice were bled, and the blood samples were pooled; blood was collected at each of the following times after injection: within 1 min; at 4, 8, 12, 20, 30, 40, and 60 min; and at 2, 4, 8, and 24 h. The animals were asphyxiated to unconsciousness with CO₂ prior to opening the chest and bleeding from the heart.

Six CD₁ rats (weight, 190 to 200 g each) were injected via the tail vein. Blood samples of about 100 μ l from each animal were obtained by tail bleeding and pooled at each of the following times after injection: within 1 min; at 5, 8, 12, 20, 30, 40, and 60 min; and at 2, 4, 6, 8, and 24 h.

Blood samples were obtained from four beagle dogs (body weight, 9.9 to 10.8 kg) at 5, 10, 15, 20, 30, 45, and 60 min and 2, 3, 4, 6, 8, 10, 12, and 24 h after drug administration. Individual samples were kept separate and were not pooled as was done for the rodents.

Blood samples were obtained from four baboons (weight range, 14 to 20 kg) at 5, 10, 15, 20, 30, and 60 min and 2, 3, 4, 6, 8, and 24 h after drug administration. Individual samples were kept separate.

In a multiple-dose study in baboons, Ro 23-9424 was administered intravenously at a dose of 20 mg/kg in 5% glucose to three baboons (weight range, 14 to 27 kg) once a day for 14 days. On the first day, the animals were not properly dosed, because we attempted to use 5% glucose in saline, in which the drug is not very soluble. On days 2, 7, and 14, samples were obtained prior to and at the following times after dosing: 5, 10, 20, 30, and 60 min and 2, 3, and 6 h. On other days, only trough and peak samples were taken. Samples were treated as described above.

Assay. The reverse-phase, ion-paired high-pressure liquid chromatography procedure was a modification of the method of Cuisnaud et al. (6). The mobile phase consisted of 0.01 M hexadecyltrimethylammonium bromide in 69% phosphate buffer (pH 8.2)–31% acetonitrile. The phosphate buffer consisted of 7.5 g of KH₂PO₄ and 2.5 g of Na₂HPO₄ · 7H₂O per liter of water; this was adjusted to pH 8.2 with 1 N NaOH. The column was a Hamilton PRP-1 (250 by 4.1 mm) equipped with a PRP-1 3-cm Fast LC guard cartridge. The flow rate was 1 ml/min, and the eluate was monitored by a UV detector at 290 nm (0.02 absorbance units, full scale). As shown in Fig. 1, fleroxacin eluted at about 6.3 min and Ro 23-9424 eluted at 11.4 min. Samples, which were stored as deproteinized supernatants containing 50% acetonitrile, were further diluted with an equal volume of 0.01 M hexadecyltrimethylammonium bromide in phosphate buffer before injection. In most cases, a refrigerated automatic sample injector (WISP; Waters Associates, Inc., Milford, Mass.) was used, and the temperature of the sample compartment was maintained at 10°C. Under these conditions, there was no degradation of Ro 23-9424 in standard solutions prepared as described below during an overnight run.

The sensitivities of the assay were approximately 0.5 μ g/ml for fleroxacin and 1 μ g/ml for Ro 23-9424. Linearity was good up to about 100 μ g/ml. For Ro 23-9424 at 10 μ g/ml, the within-run coefficient of variation was 1.7% and the between-run coefficient of variation was 2.5%. Repeated determinations of a sample spiked to contain 10 μ g of Ro 23-9424 per ml yielded a mean of 10.04 μ g/ml with a standard deviation of 0.19 μ g/ml. Standard curves were based on solutions in the respective plasma spiked to contain 1, 10, and 100 μ g of Ro 23-9424 per ml and were then treated exactly as the plasma samples were. Urine samples were

FIG. 1. Chromatograms of dog plasma samples. (A) Control. (B) Dog treated intravenously with 20 mg/kg of Ro 23-9424 per kg. Fleroxacin eluted at 6.3 min, and Ro 23-9424 eluted at 11.4 min. This sample contained 2.2 μ g of fleroxacin per ml and 6.7 μ g of Ro 23-9424 per ml.

diluted 10-fold with the mobile phase and assayed by the same method described above. The sensitivities in urine were about 10 μ g/ml for Ro 23-9424 and 5 μ g/ml for fleroxacin.

Pharmacokinetic analysis. The plasma concentration data were fitted to a two-compartment open model by using a curve stripping and nonlinear least-squares fitting program (RSTRIP; Micromath Scientific Software, Salt Lake City, Utah) to obtain the parameters of the equation $C_p = Ae^{-\alpha t} + Be^{-\beta t}$, where C_p is drug concentration in plasma, A is the zero time intercept for α phase, B is the zero time intercept for β phase, and t is time. A weighting factor of $1/y$ was used for stripping (RSTRIP uses a weighting factor of y^2 for fitting single exponentials to residuals). The area under the plasma-concentration-versus-time curve from 0 h to infinity ($AUC_{0-\infty}$) was obtained by conventional trapezoidal summation and extrapolation methods. The elimination half-life was calculated by dividing $\ln 2$ by β (the elimination rate constant). The volume of distribution was calculated by dividing the dose by the product of β and $AUC_{0-\infty}$. The systemic clearance rate was calculated by dividing the dose by $AUC_{0-\infty}$.

RESULTS

Stability. Ro 23-9424 was found to be somewhat unstable in aqueous solution, with half-lives at 37°C of 2.7 h in 10 mM phosphate buffer (pH 7.4) and 3.4 h in Mueller-Hinton broth, for example. The compound was found to be somewhat more stable in serum, with half-lives of 4.5, 3.9, 5.6, and 6.3 h at 37°C in rat, dog, monkey, and human sera, respectively. In whole blood from rats and dogs, the half-lives were similar to those in the respective sera (5.8 and 3.4 h). Plasma samples showed no degradation when they were treated and stored as described in Materials and Methods for up to 2 years.

Protein binding. Ro 23-9424 was not highly bound in sera from the species tested, and binding was independent of drug concentration from 3 to 90 μ g/ml. Mean binding percentages

FIG. 2. Concentrations of Ro 23-9424 (○) and fleroxacin (●) in pooled plasma samples from mice. Traces (about 0.3 $\mu\text{g/ml}$) of fleroxacin were detected at 4 and 8 h.

were as follows: mouse, 66%; rat, 76%; dog, 52%; monkey, 72%; and human, 66%.

Single-dose pharmacokinetics in animals. Pharmacokinetic profiles of Ro 23-9424 were determined in mice (Fig. 2), rats (Fig. 3), dogs (Fig. 4), and baboons (Fig. 5) after a single intravenous dose of 20 mg/kg. Pharmacokinetic parameters for Ro 23-9424 in the four species are summarized in Table 1. Comparative data for cefotaxime are also shown.

Concentrations of fleroxacin in plasma were low (about 1 $\mu\text{g/ml}$) but persistent. In mice, fleroxacin concentrations fell below the detection limit at 1 h, but trace concentrations were found at 4 and 8 h (data not shown). Fleroxacin was detectable up to 8 h in the rat and 24 h in the dog and the baboon (24 h time point not shown). The relatively rapid disappearance of Ro 23-9424 from the plasma without a corresponding increase in fleroxacin concentration suggested a model in which Ro 23-9424 is primarily eliminated by excretion of the intact molecule into the urine or the bile (as is typical of cephalosporins), rather than by hydrolysis to desacetylcefotaxime and fleroxacin.

Such a model would predict that the intact molecule should be found in the urine, feces, or both. Under the conditions of these experiments, a quantitative analysis of urinary and fecal recovery of drug was not attempted. However, baboon urine was collected during the first 8 h and

FIG. 3. Concentrations of Ro 23-9424 (○) and fleroxacin (●) in pooled plasma samples from rats. Traces (about 0.3 $\mu\text{g/ml}$) of fleroxacin were detected at 4, 6, and 8 h.

FIG. 4. Mean concentrations of Ro 23-9424 (○) and fleroxacin (●) in plasma from dogs. Error bars show standard errors of the mean. Traces (about 0.3 to 0.6 $\mu\text{g/ml}$) of fleroxacin were detected in all four animals at 24 h.

8 to 24 h after dosing. Concentrations of Ro 23-9424 in the range of 96 to 375 $\mu\text{g/ml}$ were found in the samples obtained from 0 to 8 h and concentrations of 29 to 63 $\mu\text{g/ml}$ were found in the samples obtained from 8 to 24 h. In another experiment, Ro 23-9424 was administered to mice (20 mg/kg, intravenously) under light methoxyflurane anesthesia. Under these conditions, the animals do not urinate and urine was aspirated from the bladders at various times. As shown in Table 2, concentrations of intact Ro 23-9424 in urine were high and generally exceeded those of fleroxacin.

Multiple-dose pharmacokinetics in baboons. Ro 23-9424 (20 mg/kg, intravenously) was administered once daily for 14 days. On days 2 (the animals were not properly dosed on day 1), 7, and 14, samples for a complete plasma profile were obtained. On other days, only trough and peak samples were taken. The results for days 2 through 14 are summarized in Fig. 6. On day 4, concentrations of both Ro 23-9424 and fleroxacin in plasma were lower than expected, but with this exception, the peak and trough levels of both drugs were quite reproducible from days 3 to 14. Trough levels of Ro 23-9424 were undetectable, while peak levels averaged 142 $\mu\text{g/ml}$ on days 5 through 14. Trough levels of fleroxacin averaged 0.4 $\mu\text{g/ml}$ and peak levels averaged 2.9 $\mu\text{g/ml}$. The

FIG. 5. Mean concentrations of Ro 23-9424 (○) and fleroxacin (●) in plasma from baboons. Error bars show standard errors of the mean. Traces (about 0.2 to 0.4 $\mu\text{g/ml}$) of fleroxacin were detected in all four animals at 24 h.

TABLE 1. Pharmacokinetic parameters of Ro 23-9424 compared with those published for cefotaxime^a

Species and drug	AUC _{0-∞} (μg · h/ml)	t _{1/2β} (min)	V (ml/kg)	CL _S (ml/h per kg)
Mouse				
Ro 23-9424	7.4	13	812	2,696
Cefotaxime ^b	NA ^c	(18) ^d	NA	NA
Rat				
Ro 23-9424	19	17	433	1,063
Cefotaxime ^e	14	15 ^f	528 ^f	1,481 ^f
Dog				
Ro 23-9424	45	36	390	449
Cefotaxime ^g	(82) ^d	44 ^f	673 ^f	630 ^f
Cefotaxime ^h	41	43 ^f	503 ^f	488 ^f
Baboon, Ro 23-9424	75	75	468	268
Human, cefotaxime ⁱ	97 ^j	62 ^f	326 ^k	204 ^k

^a Abbreviations: AUC_{0-∞}, area under the plasma concentration-versus-time curve from 0 h to infinity; t_{1/2β}, elimination half-life; V, volume of distribution; CL_S, systemic clearance rate.

^b Data from reference 14; subcutaneous administration.

^c NA, Not available.

^d Results are shown in parentheses when the published data are not directly comparable because of differences in dose or route of administration.

^e Data from reference 16.

^f Results recalculated from published values.

^g Data from reference 10; dose, 50 mg/kg.

^h Data from reference 17.

ⁱ Data from reference 7; values calculated from all studies (doses of 500, 1,000, and 2,000 mg per subject) were used.

^j Recalculated for a dose of 20 mg/kg, using the formula AUC_{0-∞} = 0.069 × dose (7).

^k Recalculated assuming that subjects weighed 70 kg.

highest peak concentration of fleroxacin in any individual sample was 4.9 μg/ml.

DISCUSSION

Results of this study suggest a model in which Ro 23-9424 is primarily eliminated by excretion of the intact molecule (as is typical of cephalosporins), rather than by hydrolysis to desacetylcefotaxime and fleroxacin. The validity of this model could be tested by analyzing the recovery of the intact molecule and its metabolites in the urine and feces. Given the difficulty of collecting animal excreta under the experimental conditions and the suspected instability of the drug under those conditions, no attempt was made to quantitate the total recovery of Ro 23-9424 in the excreta. The concentrations found in baboon and mouse urine, however, were sufficient to indicate that urinary excretion of the intact molecule is a major route of elimination of Ro 23-9424, as it

is for cefotaxime. Ro 23-9424 has been chosen for clinical trials, and this hypothesis will soon be tested in humans in whom urine collection is logistically simpler than it is in animals.

Cefotaxime is the logical compound for pharmacokinetic comparison with Ro 23-9424, since it has the same 7-acylamino substituent and is also esterified at the 3' oxygen. Cefotaxime is known to be deesterified in vivo to form desacetylcefotaxime, which may be further metabolized (5). Cefotaxime is stable in whole blood, but is degraded by hemolyzed blood and tissue homogenates, forming desacetylcefotaxime and other metabolites (5). Nevertheless, 40 to 60% of an intravenously administered dose in humans may be recovered as the unchanged molecule in urine (7).

The pharmacokinetic similarities of the two compounds are illustrated by the data in Table 1, in which the pharmacokinetic parameters of Ro 23-9424 are compared with those of cefotaxime. Surprisingly, there is very little published data on the pharmacokinetics of cefotaxime in mice. The half-life of 18 min reported for cefotaxime by Murakawa et al. (14) after subcutaneous administration is, however, consistent with the 13-min half-life after intravenous administration of Ro 23-9424 in the present study.

In rats, the pharmacokinetic parameters of Ro 23-9424 are quite similar to those reported for cefotaxime by Omosu et al. (16), except that the systemic clearance rate for cefotaxime is somewhat greater than that for Ro 23-9424 (Table 1).

Two studies of cefotaxime pharmacokinetics in dogs have appeared (10, 17). The results of Togashi et al. (17) are very similar to those of the present study (Table 1). Guerrini et al. (10) used a higher dose (50 mg/kg), so their results for AUC_{0-∞} and other dose-dependent variables are higher than both ours and those of Togashi et al. (17). The half-life reported by Guerrini et al. (10) is almost identical to that reported by Togashi et al. (17) and similar to our value for Ro 23-9424, but the values of the volume of distribution and the systemic clearance rate obtained by Guerrini et al. (10) are somewhat higher than either ours or those of Togashi et al. (17) (Table 1).

Unfortunately, no directly comparable pharmacokinetic data have been reported for cefotaxime in baboons. On the premise that both baboons and humans are large primates, however, it is interesting to compare our results for Ro 23-9424 in baboons with the pharmacokinetic parameters of cefotaxime in humans. The results reported by Esmieu et al. (7), which summarized numerous studies of cefotaxime in humans, are in fact quite comparable to our own for Ro 23-9424 in baboons (Table 1).

We have presented results for only one of the putative metabolites of Ro 23-9424, namely, fleroxacin. After several unsuccessful attempts to develop an assay method that would detect Ro 23-9424, fleroxacin, and desacetylcefotaxime simultaneously, we decided to proceed with the assay described here, for the following reasons. First, fleroxacin presumably can be formed from Ro 23-9424 in vivo by two routes: hydrolysis of the ester bond and hydrolysis of the β-lactam amide bond. Desacetylcefotaxime would be formed by the first reaction, but not by the second. Thus, the amount of desacetylcefotaxime formed in vivo can be no more than the amount of fleroxacin. Second, fleroxacin is relatively stable metabolically and has a much longer pharmacokinetic half-life than desacetylcefotaxime (at least in humans [11, 18]). Third, desacetylcefotaxime generally has much lower antibacterial activity than fleroxacin (except against a few species, notably, streptococci). In short, (i) desacetylcefotaxime is the less important metabolite, and (ii)

TABLE 2. Concentrations of Ro 23-9424 and fleroxacin in urine of anesthetized mice

Time (min)	Concn (μg/ml)	
	Ro 23-9424	Fleroxacin
10	306	47
20	328	32
30	630	80
40	498	75
60	438	90
120	414	132

FIG. 6. Mean concentrations of Ro 23-9424 (○) and fleroxacin (●) in plasma from baboons in the multiple-dose study. Note the change in the abscissa for data from days 2, 7, and 14.

an upper limit can be placed on its concentration without assaying it directly. Therefore, desacetylcefotaxime concentrations were clearly of secondary interest.

We did assay for desacetylcefotaxime using a modified assay method in one set of rat samples (data not shown). Except for the first sample (taken as soon as possible after drug administration), which contained 1.3 µg of desacetylcefotaxime per ml, only traces of desacetylcefotaxime were found, and these disappeared completely after 12 min. Although this assay method was not fully validated, the results do support the rationale given above.

Some fleroxacin is apparently formed from Ro 23-9424 *in vivo*. Since the half-lives of Ro 23-9424 and fleroxacin are substantially different—17 min versus 2.65 h (13) in the rat, for example—it appeared possible that levels of fleroxacin in plasma might accumulate to unacceptable levels on repeated dosing with Ro 23-9424. The multiple-dose study in baboons showed only a slight accumulation of fleroxacin, however. Peak fleroxacin concentrations were no higher than 4.9 µg/ml, which is comparable to that found in humans after a normal oral dose of 400 mg of fleroxacin (18, 20). The dose regimen in this multiple-dose study was only once daily to avoid undue stress to the animals. Somewhat greater accumulation of free fleroxacin may be expected at the anticipated human dosing frequency of two or three times daily.

Although some fleroxacin is produced *in vivo*, Ro 23-9424 must be considered as a therapeutic agent in its own right, not simply as a prodrug, just as cefotaxime is considered to be a therapeutic agent. Unlike cefotaxime, however, one of

the metabolic products of Ro 23-9424, fleroxacin, is at least as active as the parent molecule against many strains of bacteria.

The low but persistent concentrations of fleroxacin produced metabolically may have significant therapeutic effects and must also be considered in analyzing the *in vivo* activity of Ro 23-9424. The persistence of fleroxacin after elimination of Ro 23-9424 is a phenomenon that requires further investigation. A reasonable working hypothesis is that there is some biliary excretion of Ro 23-9424. Drug excreted in the bile would be hydrolyzed in the gut to fleroxacin and desacetylcefotaxime or other by-products. The fleroxacin so formed could then be reabsorbed from the gut.

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