Urinary and Biliary Excretion of the 2,4-Diaminoquinazoline Antifolate, Methasquin, in Rats and Dogs

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SUMMARY

The urinary and biliary excretion of methasquin [N-(p-][2,4-diamino-5-methyl-6-quinazolyl)methyl]amino]benzoyl]-L-aspartic acid, a new quinazoline antifolate, has been studied in rats and dogs. A microbiological assay utilizing Streptococcus faecium var. durans as the test organism has been developed and permits the measurement of methasquin levels in serum, bile, and urine following i.v. and p.o. administration of the antifolate. Renal clearance and serum protein binding of methasquin have been determined in the dog.

In both species studied, methasquin was rapidly excreted in the urine following i.v. administration of the drug. Most of the excretion, about 30% in rats and 40 to 50% in dogs, occurred in the first 5 hr following injection. Cumulative recoveries of less than 1% (rats) and less than 6% (dogs) in the urine 48 hr after p.o. administration of the drug showed that methasquin was poorly absorbed from the gastrointestinal tract. During the first 4 to 5 hr after i.v. doses in rats, 16 to 38% of the antifolate was recovered in bile. Serum levels following administration of p.o. and i.v. doses to rats and dogs were also determined.

Studies in dogs showed that the rate of renal clearance of the agent is 0.6 to 0.7 that of creatinine. Since methasquin is about 50% bound by serum protein, the rate of renal clearance of free drug is close to that of glomerular filtration.

INTRODUCTION

The report by Hutchison (6) has detailed the biological activities of a series of 2,4-diaminoquinazoline antifolates in bacterial, cell culture, and mouse leukemia systems. Additional aspects, such as inhibition of dihydrofolate reductase (1, 3, 9, 10), dose schedules and maximal antileukemic activities (8, 13), and quinazoline-resistant sublines of L1210 mouse leukemia (7, 12), have also been studied.

One of the more effective compounds of this series, MQ,2 (NSC 122,870, prepared in the laboratories of Dr. John Davoll and Dr. John Dice of Parke, Davis and Company, Ann Arbor, Mich., and supplied by Dr. Harry B. Wood of the Cancer Chemotherapy National Service Center) has been selected for clinical trial. Toxicological studies of this compound in mice, rats, dogs, and monkeys have been carried out by Philips et al. (11).

This report describes the excretion of MQ in rats and dogs following i.v. and p.o. administration of various doses of the drug. A microbiological assay for MQ has been used for determination of levels of the antifolate in serum, urine, and bile.

The results obtained have been compared with previous studies of the fate of MTX.

MATERIALS AND METHODS

Paper Chromatography and Bioautography. Comparison of the Rf's of the inhibitory activity in the body fluids of drug-treated rats with the Rf of MQ itself was performed by the bioautographical technique described previously (4, 6). Folic acid assay medium (Difco Laboratories, Inc., Detroit, Mich.) supplemented with 0.5 mg/ml folic acid and 2% agar was used for the bioautogram plates. The solvent system was 1% K2HPO4. As little as 2 μg of MQ can be detected by this technique.

In experiments with dog samples, identification of MQ on paper chromatograms was confirmed by UV light visualization rather than by the bioautographical technique. Although this technique is less sensitive than bioautography, 5 to 10 μg of MQ can be detected.

Microbiological Assay for MQ in Body Fluids. The concentrations of MQ in body fluids of rats and dogs were determined by a microbiological disc assay procedure with S. faecium var. durans as the test organism.

Assay plates were prepared with Difco folic acid assay broth supplemented with 0.5 mg/ml folic acid and 2% Difco Bacto-agar. The medium was autoclaved as directed and cooled to 45-50° in a water bath. Cultures of S. faecium var. durans were grown to the early stationary phase (7 to 8 hr, 37°) in Difco folic acid assay broth supplemented with 2 μg/ml folic acid, were washed twice with cold 0.85% NaCl solution and were added to the cooled medium to give a final concentration of 3 X 106 cells/ml. The inoculated medium was distributed into 13-cm flat-bottomed Petri dishes (12 ml/dish), and the plates were allowed to harden. Plates were inverted and stored at 4° for no more than 1 week.

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Antibiotic Zone Reader. Zone diameters for each of the 3
N.H. mg/ml (stored at 4°) for each assay. Dilutions of a “stable
sample” of MQ of known standard concentration of 300 µg/ml
were also prepared for each assay. Samples of body fluids
(urine, serum, bile) were diluted with water. A volume of 0.02
ml of each solution to be assayed was applied to a filter paper
disc (6.35-mm diameter) (Schleicher and Schuell, Inc., Keene,
N. H.).

Eight replicate discs were used for each standard solution; stable sample dilutions and unknown samples were tested in
triPLICATE at each of 3 different dilutions. A dilution of 1:10
was sufficient to remove interference from stimulatory or
inhibitory factors present in the fluids assayed. All discs were
allowed to air dry (0.5 to 1 hr, 37°) and were then placed on
the seeded plates. Plates were incubated at 37° for 16 to 18 hr,
and zone diameters (in mm) were determined with a
Fisher-Lilly (Fisher Scientific Co., New York, N. Y.)
Antibiotic Zone Reader. Zone diameters for each of the 3
standard curve dilutions were averaged, and a plot of zone
diameter versus concentration was drawn (Chart 1). Standard
sample zones were measured, corrected for dilution, and
compared with the known concentration of the sample. This
internal check was used as an indication of the accuracy of the
standard curve. An error of less than ±10% was considered an
indication of a suitable standard curve. Zones of all unknown
samples were measured, and the amount of MQ present was
determined from the standard curve.

The results presented represent averages of at least 2
separate assays, with triplicate determinations from each of 3
dilutions within each assay.

Rats. The rats used were adult CD males, 230 to 420 g
(Charles River Breeding Laboratories, Wilmington, Mass.). They were either given injections of MQ i.v. in femoral veins,
which had been exposed by small skin incisions while the
animals were lightly anesthetized with ether, or treated p.o. by
intragastric intubation. For injection, MQ was dissolved in
0.9% NaCl solution by addition of slightly less than 2 m
equivalents of NaOH; the resulting solutions (pH 8 to 9) were
given in the constant volume of 0.01 ml/g body weight.

For studies of urinary recovery, rats, after being deprived of
food overnight, were given by intragastric intubation a
solution containing 5% dextrose and 0.3% NaCl, 0.01 ml/g.
Thirty min later, they received different doses of MQ either
i.v. or p.o. while controls received 0.9% NaCl solution. Each
rat was then placed in a metabolism cage, given no food but
permitted to drink freely of the 5% dextrose-0.3% NaCl
solution. Urine was collected in flasks immersed in ice. Bile
samples were obtained from rats, anesthetized with
pentobarbital sodium, through polyethylene cannulas that had
been inserted into common bile ducts. A 30-min control
collection was obtained from each animal before it was given
an injection of MQ; hourly samples were taken thereafter. Bile
flow ranged from 1 to 2 ml/hr. All samples were collected in
tubes immersed in ice. Serum was obtained from rats at
various times after different i.v. doses of MQ. For this purpose,
the animals were anesthetized with ether, and blood was
drawn from the abdominal aorta. All samples of urine, bile,
and serum were stored at −20° until they were analyzed.

Dogs. Adult mongrel female dogs were used for studies of
serum concentration and urinary recovery at various times
after i.v. or p.o. administration of MQ. On the day before
dosing, the animals were lightly anesthetized with Pentothal
sodium i.v.; and their bladders were catheterized, drained, and
rinsed with sterile 0.9% NaCl solution. They were then placed
in metabolism cages and given free access to water but no
food; control urine was collected for 24 hr in flasks immersed
in ice. At the end of this period, they were again anesthetized
with pentothal and their bladders were drained and rinsed as
above. Thirty min later, after the dogs had recovered from
anesthesia, control blood samples were taken from jugular
veins. The animals were then given MQ, 1 mg/kg either in
gelatin capsules p.o., or i.v. in solution (0.5 ml/kg). At various
times thereafter, blood samples were obtained and urine was
collected by bladder catheterization as described above. Two
additional dogs, a female and a male, were given MQ, 0.1
mg/kg i.v., for determination of serum concentrations. Urine
was not collected from this pair and they were not starved. All
samples of urine and serum were stored at −20° until they
were analyzed.

Renal Clearance. The simultaneous renal clearance of MQ
and CR was studied in female dogs anesthetized with
pentobarbital sodium. The bladder of each dog was
catheterized, and the animal was hydrated by i.v. infusion of
5% dextrose, 25 ml/kg. It was then given a primer dose of CR,
100 mg/kg i.v., and a continuous i.v. infusion was begun of
0.4% CR in 5% mannitol at a constant rate of 4 ml/min. A
control 10-min collection of urine was obtained. At the end of
this period (as well as of all subsequent collections), the
bladder was rinsed with sterile 0.9% NaCl solution, and the
rinse was included in the urine sample. During the control
period, a control sample of blood was obtained from the
jugular vein. MQ, 0.1 ml/kg, was then injected i.v. Thirty min
later, 4 successive 10-min collections of urine were begun.
Blood samples were taken at 2 min before the midpoint of
each of these 10-min periods for the determination of CR and

Chart 1. Standard curve for MQ. Each symbol is the value for a single
zone of inhibition. See text for details of the assay procedure.

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MQ in serum. The values thus obtained were used for calculation of clearances during each period, respectively.

**Serum Protein Binding.** Binding of MQ by dog serum was studied by ultrafiltration through dialyzing membranes. For this purpose, 5-ml samples of dog serum were diluted with 0.5 ml of a solution of MQ (11 µg/ml) in 0.9% NaCl, and the mixtures were introduced into small bags prepared from dialyzing tubing. To obtain ultrafiltrates, the bags were centrifuged for 2 to 4 hr at 4° in Toribara tubes (14) containing an atmosphere of 5% CO₂ - 95% O₂. Control ultrafiltrates were also prepared with samples of the same sera to which 0.9% NaCl solution had been added. The capacity of the dialyzing tubing to retain MQ was checked by analysis of filtrates prepared by centrifugation of mixtures of 5 ml of 0.02 M NaHCO₃ and 0.5 ml of MQ (11 µg/ml) in 0.9% NaCl solution.

**RESULTS**

**Identification of MQ in Body Fluids.** Five-hr collections of urine from 4 rats receiving 100 mg/kg i.v. had UV absorption spectra in 0.1 N NaOH and 0.1 N HCl indistinguishable from that of MQ. Similarly, spectra (in 0.1 N NaOH) of bile collections from 2 rats given 100 mg/kg i.v. were indistinguishable from that of MQ. In addition, the inhibitory activity in the body fluids of rats and dogs was identified as MQ by paper chromatography and/or bioautography as described.

**Excretion of MQ in Rat Urine.** Chart 2 shows the cumulative recovery (% of total dose) of MQ from the urine of rats receiving i.v. doses of 100, 10, and 1 mg/kg and p.o. doses of 100 and 10 mg/kg. Between 31 and 35% (range, 23 to 43%) of all 3 i.v. doses were recovered in the urine within 5 hr after administration of the drug. An additional 1 to 4% of these doses was recovered between 5 and 24 hr after injection, and 0.2 to 1% appeared between 24 and 48 hr. The cumulative percentage of recovery was 37% at 100 mg/kg, 34% at 10 mg/kg, and 36% at 1 mg/kg.

In contrast, when MQ was administered p.o. to rats at doses
of 100 or 10 mg/kg, less than 0.2% of the total dose appeared in the urine during the first 5 hr. During the 5- to 24-hr period, between 0.06 and 0.9% of the p.o. doses appeared in the urine; an additional 0.04 to 0.6% was recovered between 24 and 48 hr. In all p.o.-treated rats, the cumulative percentage recovery by 48 hr was less than 0.7% of the administered dose.

Excretion of MQ in Rat Bile. Chart 3 shows the cumulative recovery (percentage of total dose) of MQ from the bile of rats given i.v. injections of 100, 10, and 1 mg/kg of the antifolate. At 100 mg/kg, 4 to 5% of the injected dose appeared in the bile by the end of the 1st hr. Continued excretion during the subsequent 4 hr resulted in a cumulative recovery of 37 to 39% of the total dose. At doses of 10 and 1 mg/kg, the percentage recovered rose steadily during the 1st 4 and 5 hr, resulting in an accumulation of 16 to 24% of the total dose.

It is apparent from the slopes of cumulative % recovery curves in Chart 3 that bile levels of MQ were still increasing 4 and 5 hr following administration of all 3 doses. Probably, more MQ would have been recovered from the bile had the collections been continued. The highest levels of MQ in bile (2475 to 2575 µg/ml) were reached 2 to 3 hr after administration of the 100 mg/kg dose. Three to 5 hr following the 10 and 1 mg/kg doses, bile MQ concentrations reached their highest levels at 130 to 180 and 14 to 27 µg/ml, respectively.

Serum Levels of MQ in Rats. Chart 4 shows the changes in serum levels of MQ in rats given i.v. doses of 100, 10, and 1 mg/kg. At all 3 doses, serum levels dropped sharply during the 1st hr to levels of 20 to 25% of those measurable 10 min after administration of the antifolate. Serum levels declined more slowly in the following 3 hr, decreasing each hr by one-third to one-half of the previous value.

Excretion in Dog Urine. In dogs, the antifolate MQ has a toxicity equivalent to that of aminopterin (11). Because of this marked difference in toxicity between the rat and the dog, MQ doses for the latter were considerably reduced; i.e., to 1 and 0.1 mg/kg.

Table 1 shows the urinary excretion of MQ in dogs following the administration of a dose of 1 mg/kg i.v. and p.o. Within 5 hr following i.v. administration of this dose, 41 to 50% of the drug appeared in the urine. An additional 20 to 30% appeared in the 5- to 24-hr period, resulting in a total recovery of 61 to 80% of the administered dose. Henderson et al. (5) have reported that in dogs given tritiated MTX, 0.5 mg/kg i.v., 65% of the label appeared in urine within 6 hr. An additional 5% was measured in the 6- to 24-hr period.

In contrast, less than 0.5% of the p.o. dose of 1 mg/kg MQ appeared in the urine in the 1st 5 hr. Small amounts (0.16 and 1.1%) appeared in the 5- to 24-hr samples, and in 1 dog 4.2% was recovered in the 24- to 48-hr sample (Table 1).

Serum Levels of MQ in Dogs. Chart 5 shows the changes in serum MQ levels in dogs following i.v. doses of 1 and 0.1 mg/kg and p.o. administration of 1 mg/kg. Each curve is the result for a single animal. The urinary recovery from the dogs given 1 mg/kg i.v. and p.o. is reported in Table 1.
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Of the 6 dogs in Chart 5, the 4 receiving i.v. doses and 1 receiving a p.o. dose died between 4 and 6 days following drug administration after a course of intoxication similar to that associated with single lethal doses [Philips et al. (11)]. The 6th dog survived without any signs of intoxication. In this animal, serum MQ levels were undetectable following the p.o. dose (see Chart 5).

**Serum Protein Binding in Dogs.** The extent of MQ binding in dogs was determined by ultrafiltration as described. Microbiological assays were performed on the ultrafiltrates. In 4 filtrations of sera to which MQ had been added to a final concentration of 1 μg/ml (in the range of serum values of dogs shown in Chart 5), the extent of serum protein binding was found to be 55, 57.5, 53.2, and 55%. Henderson et al. (5) have reported that at a concentration of 1 μg/ml, 43% of MTX is bound by serum protein in the dog.

Blakley (2) has stated that, since folate and its analogs pass through semipermeable membranes much more slowly than water, serum protein binding values obtained by the method of ultracentrifugation are too high. We have found that MQ readily passes through the dialysis tubing used in these ultrafiltration studies. In 2 control filtration experiments, the retention of MQ by the tubing was determined by assaying ultrafiltrates prepared by centrifuging 5 ml of 0.02 M NaHCO₃ and 0.5 ml of MQ (11 μg/ml in 0.9% NaCl solution). After dialysis, 90 and 96% of the MQ was measurable by the disc assay. These values are within limits of the experimental error of the assay.

**Simultaneous MQ and CR Clearance in Dogs.** Table 2 shows the results of studies in which simultaneous CR and MQ clearances were measured in dogs following the i.v. administration of a dose of MQ of 0.1 mg/kg. The clearance values (ml/min) obtained for both CR and MQ were compared and expressed as the fraction $C_{MQ}/C_{CR}$. At the 8 time intervals (4 in each of 2 dogs) compared, the ratios of the clearance of MQ to the clearance of CR were between 0.60 and 0.71.

### DISCUSSION

The microbiological assay used in these studies has several advantages over other assay procedures which have been used for measuring antifolates such as MTX and dichloro-MTX. The disc assay is specific for MQ itself. Possible breakdown or degradation products such as 2,4-diamino-6-methylquinazoline $p$-[(2,4-diamino-6-quinazolinyl)methyl]amino benzoic acid or a 5-unsubstituted compound, are biologically inactive for *S. faecium* var. *durans* in the concentration range used in the assay procedure. The disc assay is also considerably more sensitive than a spectrophotometric assay. As little as 1 mg/ml of MQ gives a measurable zone of inhibition on the assay plates. The assay is of sufficient sensitivity to measure extended retention of antifolates in tissues, as was shown for MTX by Fountain et al. (4). Werkheiser (15) demonstrated that the microbiological assay for MTX was as sensitive as the folate reductase assay in determining target enzyme activity.

The present MQ assay is 10 times more sensitive than the corresponding MTX assay (4) and is being used successfully to measure long-term MQ retention in tissues of mice.

It has been shown by Philips et al. (11) that the toxicity of MQ in rats is comparable to that of MTX. It is therefore of interest to compare the excretion of these 2 antifolates in this species. Using tritiated MTX at a dose of 25 mg/kg i.p., Henderson et al. (5) observed that 39 to 51% of the administered dose appeared in the urine by the end of 48 hr. Our results indicate that 34 to 37% of i.v. doses of MQ of 1, 10, and 100 mg/kg appear in rat urine within 48 hr.

A difference between the 2 antifolates is seen when the drugs are administered p.o. to rats. Following tritiated MTX doses of 0.5 and 25 mg/kg, 23 to 34% appears in the urine within 24 hr (5). In contrast, during the 1st 24 hr after p.o. administration of MQ at 10 and 100 mg/kg, less than 0.7% of the dose is recovered in the urine. This difference indicates that MQ is less readily absorbed from the gastrointestinal tract than is MTX. That MQ is poorly absorbed after p.o. doses has

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**Table 2**

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<th>Experiment</th>
<th>Total dose of MQ (μg)</th>
<th>Time (min)</th>
<th>Volumea (ml)</th>
<th>MQ (μg/ml)</th>
<th>Serum Time (min)</th>
<th>MQ (μg/ml)</th>
<th>$C_{MQ}/C_{CR}$</th>
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<td>63</td>
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*Includes the 10 ml of 0.9% NaCl used to rinse the bladder at the end of each collection.*

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The present MQ assay is 10 times more sensitive than the corresponding MTX assay (4) and is being used successfully to measure long-term MQ retention in tissues of mice.
also been shown in toxicity studies (11): in rats, the chronic LD_{50} for MQ administered i.p. (5 daily doses) is 1.3 to 2.5 mg/kg/day, and there is a 40-fold reduction in potency when MQ is administered by intragastric intubation (i.e., LD_{50} is 80 mg/kg/day).

A 2nd difference between the 2 antifolates is apparent in studying the excretion of the compounds in rat bile. Henderson et al. (5) have observed that, when tritiated MTX is administered i.v. to rats at doses of 20 to 40 mg/kg, the maximal rate of excretion occurs with 1 to 2 hr. At 1 and 2 hr after injection, cumulative recoveries are 18 to 38 and 41 to 52%, respectively, and the total percentage of recovery by 4 hr is 51 to 57%. However, following i.v. injection of MQ at doses 1, 10, and 100 mg/kg, excretion is maximal between 3 and 5 hr. At 1 hr after injection, only 0.6 to 5% of the doses are recovered in the bile; at 2 hr, cumulative recoveries ranged from 3.8 to 19%; and the total percentage of recovery by 4 hr was 15 to 35%. As mentioned above, the slopes of the cumulative recovery curves (Chart 3) suggest that considerably more MQ would appear in the bile with time.

Because of the poor absorption of MQ in the intestine, it is likely that biliary excretion of this antifolate is a terminal route of excretion. In the case of MTX, which is much more readily absorbed from the intestine, some enterohepatic circulation appears to occur and may be significant in maintaining plasma levels following p.o. or parenteral administration of this drug (Henderson et al. (5)).

The relationship between serum and bile levels of MQ is of interest. Levels of MQ in bile remained high while serum levels were progressively declining. For example, 2 hr after the administration of 100 mg/kg, bile levels of 1975 to 2575 µg/ml were measurable, while serum levels had reached 26 µg/ml. At 4 hr after administration of this dose, bile levels of MQ were 975 to 1637 µg/ml while serum levels had fallen to 2.2 µg/ml. Similarly, 4 hr following the administration of doses of 10 and 1 mg/kg, bile levels were 150 to 168 and 12 to 27 µg/ml, respectively. The corresponding serum levels were 0.32 and 0.4 µg/ml.

In dogs treated i.v. with 1 mg/kg MQ, 41 to 50% of the dose was excreted in the urine in the 1st 5 hr, with 20 to 30% appearing in the 5- to 24-hr interval. The urinary excretion of MQ following an i.v. dose of 0.1 mg/kg can be calculated from the data presented in Table 2. In 2 dogs, 14 and 26% of the administered dose appeared in the urine within the 1st 70 min. As in rats, the recovery of MQ was low following p.o. doses, indicating that gastrointestinal absorption is also poor in this species. The potency of MQ in dogs following i.v. administration is about 20-fold greater than following p.o. administration (11).

The determination of simultaneous MQ and CR clearances in the dog indicated that MQ is cleared more slowly than is CR. The ratios of the clearance of MQ to that of CR ranged from 0.60 to 0.71. If the ratios are corrected for serum binding, then the clearance of free MQ exceeds that of CR (C_{MQ}/C_{CR} = 1.3 to 1.6). While such ratios suggest the possibility of net tubular secretion, a more detailed study of renal secretion of MQ would be necessary to substantiate this. The present results are consistent with the conclusion that the rate of renal clearance of free MQ is close to that of glomerular filtration.

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