ABSTRACT

The pharmacokinetics of d- and l-citrovorum factor (CF) are quite different with respect to the postdistributional plasma decay rates. The natural (l) isomer had a half-life (β) of 47 ± 4 (S.E.) min compared to 143 ± 15 min for the unnatural (d) isomer. Renal clearance was the same for both isomers and was proportional to glomerular filtration rate. Urinary excretion appeared to be the only route of elimination of the d isomer, while l-CF was extensively metabolized. Consequently, the unnatural isomer accumulated in great excess over the natural isomer and its active metabolite, 5-methyltetrahydrofolate. The apparent volume of distribution was about 56% of body weight for both isomers, which indicates that they have equal access to tissue compartments. The data suggest that d-CF can compete with l-CF and 5-methyltetrahydrofolate for entry into cells. Under certain conditions, accumulation of d-CF may interfere with rescue from methotrexate toxicity.

INTRODUCTION

CF is 5-formyltetrahydrofolate, a chemically stable form of reduced folate which is used to prevent host toxicity due to MTX. It is commercially available as Leucovorin, which is chemically synthesized and consists of a mixture of the equal amounts of the diastereoisomers. It is generally accepted that only the natural (l) isomer is biochemically active (2). In most attempts to define the pharmacokinetics of CF, microbiological assays were used which measured only l-CF and its metabolites (1, 4, 6, 13). From those studies, it was reported that l-CF was rapidly converted to its active metabolite, 5-CH3-THF, and that very little CF was present in plasma after either p.o. or parenteral administration of dl-CF. In the present study, we administered d/[3H]CF and used HPLC to isolate dl-CF from its metabolites. l-CF was determined by a microbiological assay and d-CF was calculated by difference. The results of this study substantiate the finding that l-CF is rapidly cleared from plasma. In contrast, d-CF is only slowly cleared from the body and accumulates in great excess as compared to either l-CF or 5-CH3-THF. The possible consequences of the prolonged presence of the unnatural isomer are discussed.

MATERIALS AND METHODS

General Protocol. The general design of the experiments consisted of i.v. infusion of d-[3H]CF plus unlabeled carrier and [14C]inulin for 180 min. Plasma and urine samples were collected for a total of 420 min. Mongrel dogs weighing 9 to 18 kg were anesthetized with pentobarbital, and the ureters were cannulated via an abdominal incision. The right femoral vein was cannulated for infusion of 5% mannitol in water which was administered at a rate of 0.2 ml/min/kg for the purpose of maintaining glomerular filtration and urine flow during pentobarbital anesthesia. Blood pressure was monitored from the femoral artery.

[3H]CF was purified on HPLC as described below, and 0.5 to 1 mCi was added to sufficient unlabeled CF to provide the appropriate concentration in the infusate. Based on results of preliminary experiments, an infusion rate of 2 μg/min/kg was chosen to achieve plasma concentrations of l-CF in the micromolar range. Thus, the pharmacokinetic studies were conducted using a range of plasma concentrations which one would hope to achieve to rescue from 10⁻⁶ to 10⁻⁷ M MTX. The infusate also contained [14C]inulin sufficient to provide a dose of 0.01 μCi/min/kg and contained carrier inulin sufficient to provide a dose of 5 mg/μCi. The above ingredients were dissolved in 44 ml of 0.9% NaCl solution and infused into a foreleg vein at a rate of 0.233 ml/min using a Harvard infusion pump.

Blood and Urine Samples. Five-ml samples of blood were collected in heparinized tubes containing 5 mg of ascorbic acid. Ascorbic acid was used to prevent oxidation of reduced folates. Plasma was collected after centrifugation in the cold and was stored at -15°. Urine was collected in flasks containing sufficient ascorbic acid to provide a final concentration of 1 mg/ml, and aliquots were frozen.

Chemicals. Generally labeled d-[3H]CF, NSC 35950, was obtained from Dr. Robert Engle, Chemical Research Section, National Cancer Institute. Unlabeled dl-CF (NSC 35950) was obtained from Dr. Harry B. Wood, Division of Cancer Treatment, National Cancer Institute.

d-5-CH3-THF was purchased from Sigma Chemical Co., St. Louis, Mo. Other chemicals and solvents were obtained from Fisher Scientific, Pittsburgh, Pa., and were of standard laboratory grade, except for the solvents used for HPLC, which were HPLC grade.

Total 3H Radioactivity and [14C]Inulin. Aliquots of plasma and urine samples were placed in formalin-treated gelatin capsules and evaporated to dryness on a hot plate. The residue was combusted in an oxygen atmosphere, H2O and 14CO2 were collected separately, and the radioactivity was measured by liquid scintillation counting. Aliquots of the infusate were combusted, and specific activity was calculated as dpm of [3H]CF per ng added to the infusion solution.

HPLC. The HPLC separation was performed using a modification of the technique described by Montgomery et al. (7). The HPLC system consisted of a Constametric II-G infusion pump and UVIII monitor (280 nm), both supplied by Laboratory Data Control Corp., Riviera Beach, Fla. A Valco (Valco Instrument Co., Houston, Texas) CF-8-HP ax, 8-port injection value was equipped with a 100-μl loop for sample injection and a 10-ml loop which was filled with the secondary mobile phase for use in step gradient elution. The column was an Altex Lichrosorb RP-18 (10 μm, 4.6 mm x 25 cm) (Beckman Instruments, Fullerton, Calif.) in series with a Whatman precolumn filled with CO-Pell ODS (Whatman, Inc., Clifton, N. J.). The primary solvent system consisted of 5% acetonitrile in 0.1 M potassium phosphate buffer (pH 4.2). With a flow rate of 1.4 ml/min, 5-CH3-THF eluted at about 13 min. At 15 min, the secondary solvent system consisting of 11% acetonitrile in 0.1 M potassium phosphate buffer (pH 5.2) was introduced and CF was eluted at approximately 19 min.

The abbreviations used are: CF, Citrovorum factor; MTX, methotrexate; 5-CH3-THF, 5-methyltetrahydrofolate; HPLC, high-pressure liquid chromatography.

1 The designations d and l do not refer to the optical rotations of the compound but are used to distinguish between the unnatural (d) isomer and the naturally occurring and biologically active (l) isomer.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CF, citrovorum factor; MTX, methotrexate; 5-CH3-THF, 5-methyltetrahydrofolate; HPLC, high-pressure liquid chromatography.

4 The designations d and l do not refer to the optical rotations of the compound but are used to distinguish between the unnatural (d) isomer and the naturally occurring and biologically active (l) isomer.

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For analysis of plasma samples, the material injected consisted of 25 μl of plasma plus 25 μl of water containing 2.5 μg each of CF and 5-CH3-THF. The precolumn was changed after about 20 plasma injections. Urine samples (50 μl) were injected without addition of carriers. The elution volumes corresponding to the peaks for known compounds were added directly to counting vials containing 10 ml of Scintiverse (Fisher Scientific), and the radioactivity was measured using liquid scintillation counting. The concentrations of d-CF and 5-CH3-THF were calculated from the specific activity of the infused d-[3H]CF. Some samples contained a large percentage of unidentified radioactive material, most of which eluted in the first 5 min after injection. In urine samples collected in the later time points of the experiments, about 10% of the unidentified radioactivity eluted at the same time as added folic acid, which is strongly retained and elutes after CF. Rigorous identification of this peak was not pursued. The unidentified radioactivity which eluted in the first 5 min was not 1H2O, because total activity measured after evaporation of samples to dryness was the same as the total obtained when whole plasma or urine was chromatographed and counted. When known amounts of d-[3H]CF were added to control dog plasma and urine samples, the recoveries of the added radioactivity were 89 and 84%, respectively. Recoveries of added [14C]5-CH3-THF were 91 and 89% from plasma and urine, respectively.

**Pharmacokinetics of CF**

**Chart 1.** Plasma levels of total radioactivity (d), d-CF (D), I-CF (O), and 5-CH3-THF (A) in a representative experiment in which d- [3H]CF was infused at a rate of 20 μg/kg/min for 180 min. The curves for the postinfusion plasma levels of d-CF and I-CF were obtained by computer fit as described in "Materials and Methods." The other curves were fit by eye.

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<th>Table 1 Pharmacokinetic parameters in 4 dogs infused with [3H]CF at a rate of 20 μg/min/kg for 180 min</th>
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- a C₁F₀, plasma clearance.
- b C₁F₀, renal clearance.
- c C₁F₀/C₄₀, clearance ratio (C₁F₀/clearance of inulin).
- d Mean ± S.E.
- e NS, not significant.
- f Probability of no difference between d and I-CF as determined by paired t test.

**RESULTS**

The results of a representative experiment are shown in Chart 1. It is apparent that the d and L isomers of CF have quite different pharmacokinetic behaviors. Both isomers display biphasic plasma decay curves, but I-CF is much more rapidly cleared. The rapid appearance of 5-CH₃-THF after the start of infusion of CF and its persistence in higher concentrations than I-CF in the postinfusion phase is consistent with previous reports (4, 6). The pharmacokinetic parameters calculated from 4 experiments in which d- and L-CF was infused at 20 μg/kg/min are shown in Table 1. The β phase is approximately 3 times as rapid for the I isomer. This is reflected in the plasma clearance rates for the 2 isomers. The apparent volumes of distribution and renal clearances were similar for both isomers, as were the clearance ratios (clearance CF/clearance inulin).

Renal clearance of both isomers was highly correlated with inulin clearance (R² = 0.91). The renal clearance of d-CF was not significantly different from its plasma clearance, and urinary excretion appears to be the major, if not the only, route of elimination for this unnatural isomer. Furthermore, when urinary 5-CH₃-THF was assayed for biological activity using the bacteria Lactobacillus casei, it was found to be twice as active on a molar basis as d-5-CH₃-THF. This indicates that none of the radioactivity associated with 5-CH₃-THF was derived from d-CF. The plasma clearance of I-CF was about 2.5 times its urinary clearance, which indicates that nonrenal mechanisms play a major role in the disposition of the natural isomer.

As shown in Table 2, 5-CH₃-THF accounted for about 9% of the total radioactivity in plasma at 180 min and 6% at 420 min. The percentages of d-CF, I-CF, and 5-CH₃-THF in urine were similar to those observed for plasma at all time points (data not shown); however, the renal clearance of 5-CH₃-THF was less than that observed for the d and I isomers, and the clearance...
agreement with previous studies in humans in which the plasma isomer is extensively metabolized and has a terminal plasma half-life of 47 min, compared to 143 min for the d isomer. These results imply that no saturable processes are involved in the metabolism or excretion of /-CF. These observations are consistent with previous reports that d-CF may explain why CF rescue is often unsuccessful in patients with reduced renal function (3). White et al. (12) reported that carrier-mediated transport of 5-CH3-THF to rescue from MTX toxicity by competing for uptake into cells. The stereospecificity of uptake in normal tissues has not been tested. Our finding that the apparent volumes of distribution of the d and / isomers of CF are 60 and 57% of body weight, respectively, suggests that the 2 isomers have equal access to a peripheral compartment that is a primary determinant of the apparent volume of distribution. This does not necessarily imply that the isomers enter this compartment by a carrier-mediated transport system or that the compartment is identical to the intracellular sites at which CF exerts its rescue from MTX toxicity. Nevertheless, our findings suggest that uptake in normal tissues may not be stereospecific and that an excess of the unnatural isomer could interfere with the uptake of /-CF and 5-CH3-THF by normal tissues.

As shown in Table 2, the plasma concentrations of d-CF 4 hr postinfusion were 23 times as great as the concentration of /-CF and 6 times as great as that of 5-CH3-THF. Because d-CF is cleared from plasma only by renal excretion, whereas /-CF and 5-CH3-THF are metabolized, in the event of impaired renal function, d-CF would be present in even greater concentrations and 5-CH3-THF are metabolized, in the event of impaired renal function. In fact, the extensive accumulation of the unnatural isomer could interfere with the uptake of /-CF and 5-CH3-THF by normal tissues.

Walter and Berlino (8) reported rapid metabolism of radiolabeled CF. In 2 experiments, CF was infused at doses of 2 and 100 µg/kg/min to see if the pattern of metabolism was altered. As shown in Table 3, the plasma levels of total radioactivity at the end of 180 min of infusion were linear with respect to infusion rates. Furthermore, the percentages of /-CF and 5-CH3-THF after infusion of 2 or 100 µg/kg/min were comparable to the percentages observed after infusion of 20 µg/kg/min, as was the plasma clearance of /-CF. These results imply that no saturable processes are involved in the metabolism or excretion of CF over a 50-fold concentration range.

DISCUSSION

These studies clearly show that the pharmacokinetic behavior of the d and / isomers of CF are quite different. The unnatural (d) isomer appears to undergo little metabolic degradation and is excreted from the body almost entirely by urinary excretion. The renal excretion of the / isomer is similar to that for the d isomer. For both isomers, the rate of renal excretion is proportional to glomerular filtration rate as measured by inulin clearance (clearance ratio, 0.88). In addition to renal excretion, the / isomer is extensively metabolized and has a terminal plasma half-life of 47 min, compared to 143 min for the d isomer.

The observation that /-CF undergoes rapid metabolism is in agreement with previous studies in humans in which the plasma concentrations of the / isomer and 5-CH3-THF were determined using microbiological assays (1, 4, 6, 13). In addition, Nixon and Bertino (8) reported rapid metabolism of radiolabeled CF in humans. The latter study utilized tracer doses of enzymatically synthesized /-CF and therefore did not demonstrate differences between the natural and unnatural isomers.

Rothenberg et al. (10) used a radiochemical assay which does not distinguish between d and / isomers of CF and observed a very slow plasma clearance of CF in 2 human subjects. The slow clearance was tentatively attributed to accumulation of the inactive isomer. Our studies in dogs definitively demonstrate that the inactive isomer selectively accumulates after the administration of a mixture of the d and / isomers of CF. Furthermore, these studies show that rather than selective renal secretion of the active isomer (as speculated by Rothenberg et al.), the 2 isomers are handled identically by the kidney. The relative accumulation of the unnatural isomer is attributable to a lack of metabolism, whereas the natural isomer is extensively metabolized.

It is generally accepted that the unnatural isomer of CF is devoid of activity as a folate coenzyme (2). However, the biological significance of the presence of the unnatural isomer in excess of both the natural isomer and its active metabolite is unknown. It is possible that d-CF may impair the ability of /-CF and 5-CH3-THF to rescue from MTX toxicity by competing for uptake into cells. The stereospecificity of the uptake process is controversial. White et al. (12) reported that carrier-mediated transport of 5-CH3-THF was not stereospecific in Ehrlich ascites tumor cells. However, Sirotnak et al. (11) found that competition by CF for transport of MTX was stereospecific in L1210, Ehrlich, and Sarcoma 180 tumor cells. Stereospecificity of uptake in normal tissues has not been tested.

Our finding that the apparent volumes of distribution of the d and / isomers of CF are 60 and 57% of body weight, respectively, suggests that the 2 isomers have equal access to a peripheral compartment that is a primary determinant of the apparent volume of distribution. This does not necessarily imply that the isomers enter this compartment by a carrier-mediated transport system or that the compartment is identical to the intracellular sites at which CF exerts its rescue from MTX toxicity. Nevertheless, our findings suggest that uptake in normal tissues may not be stereospecific and that an excess of the unnatural isomer could interfere with the uptake of /-CF and 5-CH3-THF by normal tissues.

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Differences in the Pharmacokinetics of the Diastereoisomers of Citrovorum Factor in Dogs

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