Pharmacokinetics of the Diastereoisomers of Leucovorin after Intravenous and Oral Administration to Normal Subjects

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ABSTRACT

After i.v. administration of d,l-, 5-formyltetrahydrofolate (d,l-CHO-THF) CHO-THF was rapidly cleared from the plasma by conversion to 5-methyltetrahydrofolate (5-CH3-THF) and urinary excretion, whereas d-CHO-THF, which was not metabolized and was slowly excreted in the urine, persisted in plasma at concentrations greatly exceeding those of l-CHO-THF and 5-CH3-THF. The plasma half-life (ß) of the unnatural (d) isomer was 451 ± 24 (S.E.) min compared to 31.6 ± 1.1 min for the natural (l) isomer, and 227 ± 20 min for its active metabolite, 5-CH3-THF. The half-lives and volumes of distribution of each of the three compounds were independent of dose over a range of 25 to 100 mg, indicating that mechanisms for distribution, metabolism, and excretion are not saturable over the dose range tested.

The urinary clearance of l-CHO-THF or 5-CH3-THF differed only slightly from creatinine clearance, whereas urinary clearance of d-CHO-THF was only one-fifth creatinine clearance, indicating that d-CHO-THF was extensively reabsorbed. Absorption of d,l-CHO-THF after p.o. administration was stereoselective in that absorption of the l-isomer was approximately 5 times that of the d isomer. Thus, p.o. administration resulted in a more favorable ratio of active to inactive folates in plasma. At a dose of 25 mg, absorption approached 100% for l-CHO-THF compared to 20% for d-CHO-THF. However, absorption was saturable, and lower percentages of both compounds were absorbed at doses of 50 and 100 mg.

INTRODUCTION

Leucovorin calcium is the soluble calcium salt of CHO-THF, a chemically stable form of reduced folate which is used to treat or prevent host toxicity due to MTX. It is produced by chemical rather than enzymatic reduction and therefore consists of equal amounts of the diastereoisomers (d,l-CHO-THF). It is generally accepted that only the natural (l) isomer is active as a cofactor (1). Previous studies of the human pharmacokinetics of leucovorin have only considered the disposition of the active isomer. Nixon and Bertino (8) reported that, at 90 min after i.v. infusion of radiolabeled l-CHO-THF, greater than 60% of the radiolabel was present as its active metabolite (5-CH3-THF), whereas, after p.o. administration, almost all of the serum radioactivity was identified as 5-CH3-THF. Mehta et al. (5) administered d,l-CHO-THF and studied the disposition of l-CHO-THF and its metabolites using microbiological assays that measured only the biologically active species. Their studies confirmed those of Nixon and Bertino (8) in that l-CHO-THF administered p.o. appeared to be readily absorbed and converted to 5-CH3-THF. They also observed that, after i.m. administration of leucovorin, most of the circulating folate was in the form of 5-CH3-THF.

We reported previously that, after i.v. administration of d,l-CHO-THF to dogs, the postdistributional plasma decay rate of the inactive (d) isomer was much slower than that of the active isomer, resulting in plasma levels of the inactive isomer greatly in excess of the natural isomer or its active metabolite (12). Rothenberg et al. (9), who used a nonspecific radiochemical assay to measure leucovorin, reported that the plasma levels decayed very slowly after i.v. administration of d,l-CHO-THF to 2 human subjects.

In the studies described here, we used HPLC to separate and quantitate d,l-CHO-THF and 5-CH3-THF. A microbiological assay was used to quantitate l-CHO-THF and d-CHO-THF was calculated by difference. The pharmacokinetics of the active and inactive isomer as well as the active metabolite were studied after i.v. and p.o. administration of leucovorin at 3 dose levels.

MATERIALS AND METHODS

Subjects

Twelve normal, healthy subjects (6 males and 6 females) participated in this study after providing written consent to a protocol approved by the George Washington University Committee for Human Research. The subjects ranged in age from 23 to 38 years and weighed 52 to 84 kg.

Protocol

All subjects received leucovorin i.v. at a dose of 50 mg, and p.o. at doses of 50 and 100 mg. Six of the above subjects (5 males and one female) also received leucovorin i.v. at doses of 25 and 100 mg and 25 mg p.o. every 8 hr for a total of 9 doses.

Leucovorin calcium for p.o. administration was provided by Lederle Laboratories, Pearl River, NY, in the form of 5-mg tablets. For single p.o. doses, the subjects were instructed to fast for 8 hr before the drug was taken with 250 ml of water. Food was consumed until 4 hr after the drug was taken. Blood samples were taken prior to drug administration and at 1, 2, 3, 4, 6, 8, and 10 hr after drug administration. For the multiple-dose studies, the subjects were instructed to fast for 2 hr before and 2 hr after the drug was taken, except they were instructed to adhere to the protocol for the single p.o. doses for the final dose. During repeated administration, blood samples were taken 24, 48, and 64 hr after the first dose and, after the ninth dose, the protocol for the single-dose study was adhered to.

For i.v. administration, leucovorin (Leucovorin Calcium; Lederle) was dissolved in 10 to 20 ml of 5% dextrose (depending on dose) and infused at a rate of 2.2 or 4.4 ml/min (infusion times, 4.5 min). Blood samples were taken prior to drug administration and at 30 min and 1, 2, 4, 6, 8, 10, and 24 hr after i.v. infusion. Immediately after blood samples were collected in heparinized tubes,...
ascorbic acid (2 mg/ml of blood) was added, the tubes were centrifuged in the cold, and plasma was stored at −15°C until analyzed.

Urine was collected for 4 to 12 hr, ascorbic acid (2 mg/ml of urine) was added immediately after collection, and samples were stored at −15°C until analyzed. Aliquots of blood and urine to be used for determination of creatinine clearance were taken before addition of ascorbic acid.

Chemicals

d-L-5-CHO-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.

HPLC

HPLC separation and quantitation of 5-CH₃-THF and d-L-CHO-THF were performed using a modification of the technique described by Montgomery et al. (7). The HPLC system consisted of Waters Associates (Milford, MA) Models 6000 A and M45 solvent delivery system in conjunction with a Model 330 solvent programmer. The column was C₁₈ 5μ (Waters No. 86180 5μ) mounted in a Waters Z-module in series with a Whatman precolumn filled with CO:PeI octadecyl silane (Whatman, Inc. Clifton, NJ). Samples were injected using a Waters WISP automatic injector. The column eluate was passed through a variable wavelength UV detector (Laboratory Data Control Corp., Riviera Beach, FL) set for 300 nm. Quantitation was by the external standard method and peak integration using a Waters Data Module.

The primary mobile phase was 30% methanol and 4 mW tetrahydrofuran in water adjusted to pH 5.5 with acetic acid. The secondary phase was 60% methanol and 4 mW tetrahydrofuran in water adjusted to pH 5.5. A linear gradient (0 to 100% secondary phase) for 20 min was followed by 5 min of equilibrations with the primary phase. Retention times were approximately 13 min for CHO-THF and 14.5 min for 5-CH₃-THF. Recovery of standards added to plasma samples was in excess of 90% for both CHO-THF and 5-CH₃-THF. The lower limit of the assay was about 50 ng/ml for 200-μl injection volumes.

Sample Preparation

Acetonitrile (1.5 ml) was added to 1 ml of plasma to precipitate plasma proteins. Following centrifugation, the supernatant was removed and added to a mixture of 5.5 ml of ethyl ether and 1 ml of n-butyl alcohol, the mixture was shaken and centrifuged, and the nonaqueous layer was aspirated and discarded. The ether removed the acetonitrile, and the n-butyl alcohol reduced the sample volume by extracting a portion of the water.

Urine samples were injected directly or diluted appropriately to give peaks within the linear range of detection.

Assay for d-L-CHO-THF. The biologically active form of CHO-THF was measured in plasma and urine samples by the disc assay method of Mehta and Hutchinson (6). The test organism was a MTX-resistant strain of Pediococcus cerevisiae obtained from Dr. Bipin M. Mehta, Sloan-Kettering Institute for Cancer Research, Rye, NY.

Assay for 5-CH₃-THF. Concentrations of 5-CH₃-THF in plasma and urine were calculated as the differences between the concentration of d-L-CHO-THF measured by HPLC and d-L-CHO-THF measured by the microbiological assay.

Data Analysis

1. The plasma concentration versus time data for the elimination phases of l-CHO-THF, d-CHO-THF, and 5-CH₃-THF were fit to the equation $C = Be^{-kt}$ by the method of least-squares to calculate the elimination rate constants ($\beta$s) and $V_{iD}$ for each subject.

2. The AUCs were calculated using observed values at each time point and the trapezoid method. For i.v. infusions, the zero time concentrations of l-CHO-THF and d-CHO-THF were estimated by extrapolating a plot of the concentration versus time data, and the zero time concentration of 5-CH₃-THF was taken as zero. For both i.v. and p.o. administration, the area from the final data point to infinity was estimated as $C/\beta$, where $C$ was the concentration at the last data point.

3. Plasma clearance ($C_l$) was calculated using:

$$C_l = \text{Dose}/\text{AUC}$$

4. For calculation of $C_{lu}$ of 5-CH₃-THF, the dose used was the dose of l-CHO-THF minus the amount of l-CHO-THF excreted unchanged in the urine.

5. The apparent volume of distribution ($V_a$) was calculated using:

$$V_a = \frac{C_l}{\beta}$$

6. Urinary clearance ($C_l$) was calculated using:

$$C_l = \text{Amount in urine (0 to time t)}/\text{AUC (0 to time t)}$$

7. Only small amounts of l-CHO-THF were measurable in plasma after p.o. administration of leucovorin. Therefore, the availability of l-CHO-THF was estimated from the AUCs of its active metabolite, 5-CH₃-THF, measured after i.v. and p.o. administration of leucovorin. After i.v. administration of d-l-CHO-THF, approximately 20% (22 ± 1.7) of administered l-CHO-THF was excreted unchanged in the urine, whereas after p.o. administration, only 0.6 ± 0.12% of administered l-CHO-THF was excreted unchanged in the urine. Because that portion of a dose of l-CHO-THF excreted unchanged does not contribute to the 5-CH₃-THF AUC, the following formula was used to calculate the availability of l-CHO-THF. Dose equals the amount of l-CHO-THF administered, i.e., 50% of the dose of leucovorin.

$$F = \frac{(AUC) \text{ i.v.} \times \beta}{(AUC) \text{ p.o.}}$$

8. Statistical analysis was by analysis of variance and the Neuman-Keuls test for determinations of significance when more than 2 treatments were being tested (14). For comparison of 2 treatments in the same group, the paired $t$ test was used.

9. For the studies described here, it was necessary to make certain assumptions in order to estimate the pharmacokinetic parameters of 5-CH₃-THF and to calculate the availability of l-CHO-THF after p.o. administration of leucovorin. These assumptions were as follows. (a) For calculation of $C_{lu}$ of 5-CH₃-THF, the dose of 5-CH₃-THF was assumed to be the i.v. dose of l-CHO-THF minus the amount of l-CHO-THF excreted unchanged in the urine. (b) For calculations of the availability of l-CHO-THF, it was assumed that presystemic (after p.o. administration) and systemic (after i.v. administration) metabolism of l-CHO-THF follow the same pathways.

RESULTS

Administration i.v. As shown in Chart 1 and Table 1, the plasma half-life of l-CHO-THF after i.v. administration of leuco-
The plasma half-lives and volumes of distribution of \( l\)-CHO-THF, \( d\)-CHO-THF, or 5-CH\(_3\)-THF were the same at all i.v. doses from 25 to 100 mg (Tables 1 and 2). The consistency of these pharmacokinetic parameters indicates that the factors responsible for the distribution, excretion, and/or metabolism of these compounds are not saturable over the dose range examined.

In Table 3, the plasma clearance (\( C_l_p \)) and renal clearances (\( C_l_r \)) of the 3 compounds are compared. \( C_l_p \) of \( l\)-CHO-THF was about 4-fold more than the \( C_l_r \), indicating that elimination occurs extensively by nonrenal mechanisms, presumably by metabolism to 5-CH\(_3\)-THF. \( C_l_p \) of 5-CH\(_3\)-THF also exceeded its \( C_l_r \), indicating that metabolism (catabolism and/or anabolism) plays a major role in its disposition. In contrast, the \( C_l_p \) of \( d\)-CHO-THF was the same as its \( C_l_r \), indicating that \( d\)-CHO-THF was not metabolized or excreted by nonrenal mechanisms. This indicates that the source of 5-CH\(_3\)-THF is \( l\)-CHO-THF and that \( d\)-CHO-THF is not converted to 5-CH\(_3\)-THF. This is in agreement with our finding in dogs that 5-CH\(_3\)-THF excreted after administration of \( d\, l\)-CF had twice the biological activity of \( d\, l\)-5-CH\(_3\)-THF, indicating that the 5-CH\(_3\)-THF is derived only from the biologically active isomer (12).

Examination of Table 4 reveals that \( C_l_r \) of unbound \( l\)-CHO-THF was slightly greater than creatinine clearance. This difference is of borderline significance but indicates that the net effect of excretion and reabsorption in the kidney favors excretion. In contrast, \( d\)-CHO-THF was cleared at a rate of only one-fifth that of creatinine, and this difference was highly significant. \( C_l_r \) of unbound 5-CH\(_3\)-THF was not significantly different from creatinine clearance. Thus, the unnatural isomer (\( d\)-CHO-THF) was extensively reabsorbed, while the natural isomer (\( l\)-CHO-THF) and its active metabolite were cleared at rates approximating creatinine clearance.

**Administration p.o.** As shown in Table 5, the half-life of \( d\)-CHO-THF was the same for all doses. In contrast, the half-life of 5-CH\(_3\)-THF was significantly increased at the 25-mg dose of leucovorin.
The pharmacokinetics of diastereoisomers of leucovorin in humans was studied. After single oral doses of 50 and 100 mg, plasma clearance of 5-CH₃-THF was estimated using the Vd sat calculation. The AUCs for 5-CH₃-THF after oral administration of leucovorin were compared with creatinine clearance and renal clearance of unbound drug. The AUCs for 5-CH₃-THF after oral administration were corrected for the change in biological half-life. After repeated oral administration of leucovorin, nonrenal mechanisms accounted for 58% of the plasma clearance of 5-CH₃-THF, whereas, after repeated oral administration of leucovorin, nonrenal mechanisms accounted for only 32% of the plasma clearance. This comparison suggests that the increase in the plasma half-life of 5-CH₃-THF during repeated oral administration occurred due to a decrease in nonrenal disposition of the compound.

Table 6 shows the AUCs for 1-CHO-THF, d-CHO-THF, and 5-CH₃-THF after i.v. and p.o. administration of leucovorin. After i.v. administration, the AUCs for all 3 compounds increased in proportion to dose. However, after p.o. administration, there was considerable deviation from linearity. The failure of p.o. AUCs to increase in proportion to dose is indicative of saturation of the absorption mechanisms for d-CHO-THF and 1-CHO-THF. Because the plasma half-life of 5-CH₃-THF was different following p.o. administration of leucovorin (Table 5), it was not possible to estimate p.o. availability from the ratios of the p.o. to i.v. AUCs. The AUCs for both p.o. and i.v. administration were corrected for the change in half-life described in "Materials and Methods." This calculation not only corrects for changes in half-life associated with different experimental conditions, but also reduces intrasubject variability associated with changes in biological half-life.

The AUCs of 5-CH₃-THF reported by Lasseter et al. (4) for p.o. administration of 20 mg of leucovorin (107 ± 4) are comparable to the AUCs reported here for the single p.o. dose of 50 mg (240 ± 29) when adjusted for dose and apparent availability. This is of particular significance because the above group used leucovorin indicate that only small amounts of 1-CHO-THF escape absorption. The AUCs of 5-CH₃-THF from the 25-mg p.o. experiment. The assumption was made that Vₐ did not change during repeated administration of leucovorin. Cl, was calculated from the AUCs during the final dosing interval and the cumulative amount of 5-CH₃-THF excreted during the 8-hr period. The values obtained for Clp and CI were 61.8 ± 8.6 and 42 ± 5.4 ml/min, respectively. After i.v. administration of leucovorin, Clp and CI of 5-CH₃ were 77.5 ± 9.0 and 32.2 ± 2.0, respectively, as shown in Table 3. Thus, single i.v. doses of leucovorin, nonrenal mechanisms accounted for 58% of the plasma clearance of 5-CH₃-THF, whereas, after repeated p.o. administration of leucovorin, nonrenal mechanisms accounted for only 32% of the plasma clearance.

The AUCs of 5-CH₃-THF after p.o. administration of leucovorin indicate that only small amounts of 1-CHO-THF escape absorption. The AUCs for 1-CHO-THF after p.o. administration of leucovorin are from zero to infinity except for the 25-mg p.o. dose which was calculated for one dosing interval. The AUCs for 1-CHO-THF and 5-CH₃-THF after i.v. and p.o. leucovorin administration are shown in Table 7.
Table 7

<table>
<thead>
<tr>
<th>Dose of leucovorin (mg)</th>
<th>Mean apparent availability (mg versus mg)</th>
<th>Analysis of variance (mg)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-CHO-THF*</td>
<td>25.0 ± 0.15</td>
<td>25.50</td>
<td>NS</td>
</tr>
<tr>
<td>50.0</td>
<td>0.75 ± 0.10</td>
<td>25.100</td>
<td>0.005</td>
</tr>
<tr>
<td>100.0</td>
<td>0.37 ± 0.04</td>
<td>50.100</td>
<td>0.01</td>
</tr>
<tr>
<td>d-CHO-THF*</td>
<td>25.0 ± 0.19</td>
<td>25.50</td>
<td>NS</td>
</tr>
<tr>
<td>50.0</td>
<td>0.20 ± 0.03</td>
<td>25.100</td>
<td>0.005</td>
</tr>
<tr>
<td>100.0</td>
<td>0.067 ± 0.006</td>
<td>50.100</td>
<td>0.001</td>
</tr>
</tbody>
</table>

- Availability of I-CHO-THF was calculated from the AUC of 5-CH3-THF as described in "Materials and Methods."
- Availability of the 25-mg dose was determined during the final dosing interval after 9 doses of 25 mg every 8 hr.
- Mean ± S.E.
- NS, not significant.
- Apparent availability (F) of d-CHO-THF was calculated using:

\[ F = \frac{AUC_{p.o.} \times \beta_{p.o.}}{AUC_{i.v.} \times \beta_{i.v.}} \]

Discussion

The data presented for the i.v. administration of leucovorin substantiate previous reports that I-CHO-THF is rapidly cleared from the plasma and that its disappearance is accompanied by the appearance of 5-CH3-THF (8). We further show that this behavior is constant over a dose range of 25 to 100 mg. The much longer half-life observed for d-CHO-THF is similar to what we reported previously for dogs (12) and in agreement with the observation of Rothenberg et al. (9).

The apparent volumes of distribution of I-CHO-THF, d-CHO-THF, and 5-CH3-THF were 17.5, 7.9, and 22.9 liters, respectively (Table 2). These data suggest that the unnatural isomer (d-CHO-THF) may not distribute as extensively as the natural isomer. This conclusion is consistent with the reports that I-CHO-THF is selectively transported in preference to d-CHO-THF in murine tumor cells (10). However, one must use caution in interpreting Vd data. These values are dynamic pharmacokinetic VdS and not steady-state VdS which are more representative of physiological volumes of distribution. The volumes of distribution of d- and I-CHO-THF were not different when measured in dogs during constant i.v. infusion (12). However, we observed a species difference in the renal transport of d-CH1-OHF (see below), and other differences may exist in transport in other cells.

Studies of the renal handling of the compounds revealed some surprising results. After i.v. administration of leucovorin, unbound 5-CH3-THF was excreted at a rate approximating creatinine clearance, and unbound I-CHO-THF was excreted at a rate slightly greater than creatinine clearance. In contrast, d-CHO-THF was excreted at a rate only one-fifth of creatinine clearance, which indicates that the unnatural isomer is extensively reabsorbed. The finding that plasma clearance of d-CHO-THF was the same as renal clearance showed that d-CHO-THF was not metabolized. Therefore, the prolonged half-life of d-CHO-THF may be attributed to lack of metabolism in conjunction with limited renal excretion due to reabsorption. In consideration of the size and hydrophilicity of the CH1-OHF molecule, it is highly unlikely that d-CHO-THF is passively reabsorbed. Furthermore, folates are presumed to cross biological membrane only by active transport (1), and folic acid is reabsorbed in the kidney by a saturable, carrier-mediated system (3). Therefore, we conclude that d-CH1-OHF is actively reabsorbed in the human kidney. This is in contrast to our finding in dogs that the renal elimination of both d-CHO-THF and I-CHO-THF approximated inulin clearance (12). Furthermore, the fact that C1 of d-CHO-THF was the same at all dose levels suggests that the transport system of d-CHO-THF has a relatively high capacity which was not saturated even after an i.v. dose of 100 mg of leucovorin.

The p.o. absorption studies indicate that both d-CHO-THF and I-CHO-THF are absorbed by a saturable process and that the system is stereoselective. Absorption of I-CHO-THF was 4- to 5-fold greater than absorption of d-CHO-THF at all doses tested. Furthermore, repeated p.o. administration of leucovorin resulted in approximately equal plasma levels of 5-CH3-THF and d-CHO-THF (Chart 2), whereas, after i.v. administration of leucovorin, the concentration of the unnatural isomer greatly exceeded the concentration of the biologically active isomer (Fig. 1). The consequences of the presence of large amounts of the inactive isomer are unknown. However, d-CHO-THF is approximately one-20th as effective as I-CHO-THF in competing for uptake of MTX in murine tumor cells and only one-100th as effective as the l-isomer in preventing inhibition of growth of L1210 by MTX (10). Repeated parenteral administration would result in selective accumulation of the inactive isomer which may compete with the active isomer. It is thus possible that repeated parenteral administration of leucovorin may be less effective than p.o. administration in rescuing from MTX toxicity. These findings argue that p.o. administration of leucovorin may be preferred to parenteral administration in the treatment and/or prevention of toxicity associated with MTX treatment.

Absorption p.o. of both isomers was saturated at a dose of leucovorin of 50 mg. The fact that absorption was essentially...
complete at 2 hr after ingestion suggests that higher plasma levels of 5-CH$_3$-THF could best be achieved by frequent administration of relatively small doses rather than increasing the size of individual doses.

The observation that the plasma half-life of 5-CH$_3$-THF increased during repeated administration of leucovorin should be interpreted with caution. These studies were conducted in normal subjects, and it is likely that the increase in half-life occurred due to less active anabolic pathways for 5-CH$_3$-THF as body stores of reduced folates were saturated. In patients treated with MTX, the anabolic pathway would be expected to be maximally active due to inhibition of dihydrofolate reductase and depletion of stores of reduced folates. Consequently, the half-life of 5-CH$_3$-THF would be expected to be equal to or perhaps even shorter than observed after single doses in normal subjects. Studies in MTX-treated patients are indicated to answer this question.

REFERENCES

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