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Pharmacokinetics of reduced folates after short-term infusion of d, l-folinic acid*,**

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Summary. After the use of d,l-folinic acid (d,l-CHO-THF), pharmacokinetic measurements should take into account l-CHO-THF and its metabolite l-methyltetrahydrofolic acid (l-CH_3-THF) as well as d-CHO-THF. For this purpose, we developed a simple and rapid assay by combining reversed-phase HPLC to determine total levels of d,l-CHO-THF and CH_3-THF and chiral HPLC to separate the biologically active l-CHO-THF from the inactive d-CHO-THF. We investigated the pharmacokinetics after short-term infusion of 300 mg d,l-CHO-THF in ten healthy volunteers. With a mean of 56.5 min, l-CHO-THF exhibits a rapid body clearance of 222 ml/min, about 60% of which is caused by metabolism to CH_3-THF and 40%, by renal excretion. CH_3-THF has a terminal half-life of 208 min and a total body clearance of 88.9 ml/min, which is essentially the same as the renal clearance. Due to the lower clearance of CH_3-THF, its AUC (2,132 $\mu M \times$ min) exceeds that of l-CHO-THF (1445 $\mu M \times$ min) by approximately 50%. In contrast to that of the reduced l-folates, the total body and renal clearance of d-CHO-THF is very low, with values of 13.2 and 12.9 ml/min, respectively. This results in a very high AUC of 24, 269 $\mu M \times$ min, which is higher by factors of 17 and 11 than those of l-CHO-THF and CH_3-THF, respectively. The implications of the distinct kinetics of the reduced l-folates and d-CHO-THF for the efficacy of folinic acid/5-fluorouracil therapy and adequate protocols for the treatment of advanced colorectal cancer are discussed.

Introduction

The combination of 5-fluorouracil (5-FU) with folinic acid (*N*-5-formyltetrahydrofolic acid, leucovorin, CHO-THF) is a new concept for treating advanced colorectal cancer more effectively than with 5-FU alone [5, 18]. Promising clinical results in phase II studies [5, 9, 10] have been confirmed by several phase III studies [4–6, 15]. The biochemical rationale for this approach presumes that high doses of folinic acid increase the cellular pools of reduced folates. Conversion of l-CHO-THF to *N*-5, *N*-10-methylenetetrahydrofolate (CH_2-THF) enhances the formation and stability of the ternary FdUMP-thymidylate synthase-CH_2-THF complex, which in turn causes a profound inhibition of dTMP de novo synthesis and consequently results in impairment of DNA synthesis [3, 7, 18–20].

Commercially available folinic acid is a racemic mixture of the biologically active l-CHO-THF and the inactive d-CHO-THF [2]. Due to crucial differences in the kinetics of d- and l-CHO-THF and a rapid conversion of l-CHO-THF to l-methyltetrahydrofolic acid (CH_3-THF) [10, 11, 24, 25], pharmacokinetic studies have to consider the different kinetics of these three reduced folates. Total CHO-THF and CH_3-THF are usually separated by reversed-phase chromatography, and differentiation of l-CHO-THF from the inactive d-form is achieved microbiologically using *Pediococcus cerevisiae* [10, 24, 25] or *Streptococcus faecalis* [11]. To investigate the kinetics of d,l-folinic acid, we applied an HPLC method similar to that of Wainer and Stiffin [26], using as the first step a reversed-phase column for the separation of d,l-CHO-THF and CH_3-THF, followed by a chiral HPLC method for the determination of the two stereoisomers. To obtain kinetic data on d,l-CHO-THF under normal conditions, we investigated the kinetics after short-term infusion of 300 mg d,l-CHO-THF (the dose used in our clinical protocols for advanced colorectal cancers) in ten healthy volunteers.

Patients and methods

Treatment plan. Ten healthy volunteers consisting of six men and four women (median age, 28 years; range, 25–33 years) underwent a short-term infusion (15 min) of 300 mg d, l-CHO-THF. Venous blood samples were taken at 2, 5, 10, 20, 30, 45, 60, and 90 min and 2, 3, 7, 12, and 24 h after the end of the infusion. Urine samples were collected for 24 h in fractions over 0–2, 2–4, 4–7, 7–12, and 12–24 h. Serum and urine samples were stored at −20° C and were analyzed within 2 weeks; samples stored in the same man-

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Abbreviations: CH_2-THF, *N*-5, *N*-10-methylenetetrahydrofolic acid; CH_3-THF, *N*-5-methyltetrahydrofolic acid; CHO-THF, *N*-5-formyltetrahydrofolic acid; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine; FUH2, dihydrofluorouracil; FUPA, α-fluoro-ureidopropionic acid

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Table 1. Kinetic data of reduced folates after short-term infusion of 300 mg d, l-CHO-THF

	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	Clearance total	(ml/min): renal	$V_{d\beta}$ (l)	AUC (μM × min)
l-CHO-THF	12.6±4.0	56.5±10.5	222.5±27.1	93.6±15.3	18.3±4.9	1,445±175
CH_3-THF		208 ±70	88.9±18.1	86.1±21.9	25.8±7.9	2,132±384
d-CHO-THF	12.9±4.7	438 ±63	13.2± 1.4	13.0± 1.5	8.3±1.1	24,269± 2,626

ner with and without ascorbate showed no significant difference in CH_3-THF degradation during this period.

HPLC analysis. The method for determining d-CHO-THF, l-CHO-THF, and CH_3-THF consisted for two consecutive HPLC separations. Total d, l-CHO-THF was separated from CH_3-THF by reversed-phase HPLC (RP-HPLC), whereupon the d, l-CHO-THF peak was further separated into the d- and l-stereosiomers by means of chiral HPLC.

RP-HPLC. Using a C-18 guard column, aliquots of 20–100 μl serum were injected for separation onto a Shandon ODS-Hypersil column (5-μ*M* particle size; 4.6-mm inside diameter × 25 cm). An extraction procedure for the reduced folates was omitted. The guard column was replaced after 50 injections or when the pressure increased by 60 bar. At a flow rate of 1.2 ml/min, gradient elution of reduced folates was carried out using a Gynkotek gradient former (model 250B). Buffer A was 30 m*M* potassium phosphate (pH 7.0); buffer B consisted of 30 m*M* potassium phosphate (pH 7.0) plus 22% methanol. Elution was started isocratically with buffer A; after 4 min a linear gradient was commenced, achieving 100% buffer B over 20 min. Absorbance was monitored at 285 nm (Gynkotek UV spectral photometer SP4). Quantitative analysis was carried out using a Gynkotek Chromatopac C-3A printer-plotter by an external-standard method. Retention times of d, l-CHO-THF and CH_3-THF were 13.4 and 17.5 min, respectively. The determination of within- and between-run precision for d, l-CHO-THF showed standard deviations of ± 1.82% and ± 2.38%, respectively; the corresponding values for CH_3-THF were ± 3.91% and ± 5.29%. The detection limit was 0.1 μ*M* for both substances. The d, l-CHO-THF peak was collected and dried under a stream of nitrogen and the residue was reconstituted in 150 μl water for chiral HPLC.

Chiral HPLC. For separation of the d, l-stereoisomers of CHO-THF, we used a chiral column based on bovine serum albumin (ET 150/8/4 Resolvosil-BSA-7, 30 cm × 4-mm inside diameter; Macherey-Nagel, Düren, FRG). Aliquots of 20–100 μl were injected onto the column. Elution was carried out isocratically with 0.1 *M* sodium phosphate buffer (pH 5.0) containing 5% 1-propanol. Retention times for l- and d-CHO-THF were 21.5 and 28.1 min, respectively. Separation of d, l-CHO-THF standards showed an exact allotment of 50% to each of these two folates. The standard variation was low, with 2.03% and 1.99% for the l- and d-steroisomers respectively. Recovery of l- plus d-folinic acid after reversed-phase and subsequent chiral HPLC was 97.2% ± 7.1%. After the RP- and chiral HPLC results were combined, the detection limit of pure l-CHO-THF in specimens from test persons after d, l-CHO-THF infusion was 1 μ*M*.

Calculations. Pharmacokinetic data were calculated using standard methods [17]; the AUC was calculated according

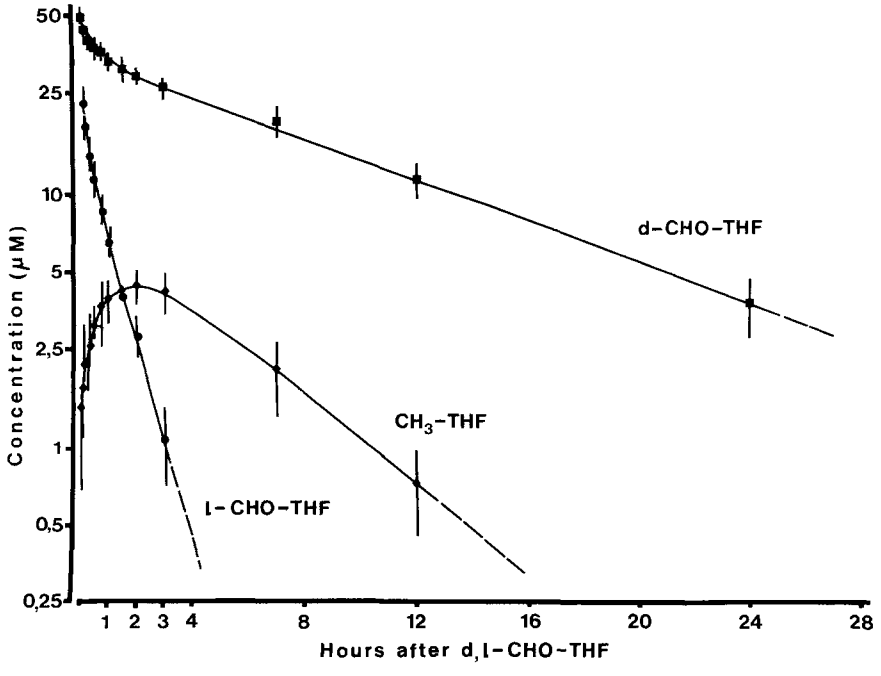

Fig. 1. Course of reduced serum folates after short-term infusion of 300 mg d, l-folinic acid in 10 healthy volunteers. Given are the mean values ± SD. *l-CHO-THF,* l-folinic acid; *d-CHO-THF,* d-folinic acid; *CH_3-THF* l-methyltetrahydrofolic aid

to the trapezoidal rule. The area of the individual trapezoids was calculated using the observed values for each time point. The area from the final measured value to infinity was estimated as C/β or $(C \times t_{1/2\beta})/0.693$. The total clearance was calculated using Cl_T = dose/AUC, and the renal clearance, Cl_R, was determined using Cl_R = total amount excreted unchanged/AUC. The volume of distribution, $V_{d\beta}$, was calculated according to $V_{d\beta} = Cl_T/\beta$ or $(Cl_T \times t_{1/2\beta})/0.693$ [17]. For calculations of CH_3-THF total clearance, we assumed that CHO-THF not excreted via the kidneys had been metabolized to CH_3-THF [24].

Results

l-Folinic acid

Kinetic data for l-CHO-THF from ten healthy volunteers are depicted in Table 1. According to the steep decrease in serum l-CHO-THF levels demonstrated in Fig. 1, short $t_{1/2\alpha}$ and $t_{1/2\beta}$ values of 12.6 min (mean) and 56.5 min (mean) were determined. l-CHO-THF peak concentrations right after the infusion were 27.5 ± 5.3 μM. After 30, 60, and 120 min, mean serum concentrations were 11.8, 6.5, and 2.8 μM, respectively (data not shown). Total body clearance was high (222.5 ± 27.1 ml/min). At 93.6 ± 15.3 ml/min, renal clearance contributed only 42% to the total body clearance. The volume of distribution ($V_{d\beta}$) was 18.3 ± 4.9 l. At $1{,}445 \pm 175$ $\mu M \times$ min, the AUC of l-CHO-THF amounted to only two-thirds of that of CH_3-THF.

Metyhl-THF

As shown in Fig. 1, serum CH_3-THF concentrations had run to the micromolar range at the end of the short-term infusion of d, l-CHO-THF. CH_3-THF serum levels increased further to peak concentrations between 4.2 and 4.4 μM after 90 and 120 min, respectively. After 3, 7, and 12 h, mean serum concentrations of CH_3-THF were 4.2, 2.0, and 0.72 μM. Kinetic data for CH_3-THF are presented in Table 1. The CH_3-THF elimination half-life was calculated as being 208 ± 70 min, and the distribution volume, $V_{d\beta}$, was 25.8 ± 7.9 l. With a total body clearance of 88.9 ml/min and a renal clearance of 86.1 ml/min, CH_3-THF was eliminated almost exclusively via the kidneys. The mean AUC of 2,132 μM exceeded that of l-CHO-THF by approximately 50%.

Total reduced l-folates

Total reduced l-folates, i.e., the sum of l-CHO-THF and CH_3-THF, measured 28.7 μM at the end of the infusion. Serum levels fell from 14.8 and 10.5 μM after 30 and 60 min to 7.2 and 5.3 μM after 2 and 3 h, respectively; thereafter, reduced l-folates were represented by CH_3-THF only. The AUC of reduced l-folates was 3,577 $\mu M \times$ min.

d-Folinic acid

At the end of the infusion, serum peak concentrations of the inactive d-steroisomer of CHO-THF were 53.1 ± 5.2 μM. They remained at high levels of >30 μM for 90 min and >10 μM for 12 h; 24 h after d, l-CHO-THF infusion, mean d-CHO-THF serum concentrations of 3.7 μM were determined (data not shown). In contrast to those of l-CHO-THF, the serum levels of d-CHO-THF decreased very slowly, with a $t_{1/2\beta}$ of 438 ± 63 min (Table 1). In accordance with the long elimination half-life, total body and renal clearance were very low, with mean values of 13.3 and 12.96 ml/min, respectively. The volume of distribution was low (8.3 ± 1.1 l). Due to the slow elimination, the AUC of the biologically inactive d-CHO-THFf was very high; values of $24{,}269 \pm 2{,}626$ $\mu M \times$ min exceeded the AUC of l-CHO-THFf 17 times and that of CH_3-THF by a factor of 11.

Discussion

Commercially available CHO-THF is a racemic mixture of the bioloically active l-CHO-THF and the inactive d-CHO-THF. Therefore, reversed-phase HPLC afforded only the separation of total d, l-CHO-THF from CH_3-THF. Until now a subsequent microbiological assay was necessary to quantitate the l-folates, especially l-CHO-THF [11–13, 19, 24, 25]. This procedure is expensive and time-consuming. In contrast to reversed-phase HPLC, chiral HPLC offers the possibility of separating the two stereosiomeric forms of CHO-THF. As did Wainer and Stiffin [26], we used a chiral HPLC column based on bovine serum albumin; these authors used 0.25 *M* phosphate buffer (pH 5.0) at a temperature of 40° C to separate the l- and d-folates. In our experiments, we used 0.1 *M* phosphate buffer (pH 5.0) with 5% 1-propanol and thus succeeded in an easy and simple separation of d- and l-CHO-THF at room temperature.

For quantitation of the CHO-THF stereoisomers, Schilsky et al. [22] also used chiral HPLC. However, they determined plasma levels of d- and l-CHO-THF as well as of CH_3-THF after oral d, l-CHO-THF administration. In patients receiving 100 mg d, l-CHO-THF q 4 h p.o., they observed very low l-CHO-THF (0.3 μM) and higher CH_3-THF levels (approximately 4 μM) [22]. Due to the poor absorption of d-CHO-THF, the plasma ratio of d-CHO-THF: l-CHO-THF + CH_3-THF was <1 [22]. These data confirm previous pharmacokinetic studies using oral d, l-CHO-THF reported by Straw et al. [24] and McGuire et al. [11].

In contrast to microbiological assays, which have a very low detection limit of 0.5–1 nM [10, 11], UV detection of folates is less sensitive; the detection limit is 0.1 μM. However, at the high d, l-CHO-THF doses of 80–500 mg/m^2 used in most clinical studies of advanced colorectal cancer [5, 6, 9, 10, 18], a lower detection limit of 0.1–1 μM is sufficient. Thus, our combined reversed-phase and chiral HPLC method yielded basic data for d- and l-CHO-THF as well as for CH_3-THf after short-term infusion of 300 mg d, l-CHO-THF in healthy volunteers. The kinetics of reduced d, l-folates during/after systemic or regional (hepatic arterial infusion) CHO-THF/5-FU therapy in patients with advanced colorectal cancer are under investigation.

Whereas Rustum et al. [19] and Straw et al. [24, 25] did not distinguish distinct phases of l-folinic acid kinetics, our investigations in healthy volunteers revealed two phases, with a distribution half-life of 11 min and an elimination half-life of 51 min. Machover et al. [10] reported $t_{1/2\alpha}$ and $t_{1/2\beta}$ values of 20 and 122 min, respectively. We cannot assess whether these higher values occurred due to the higher sensitivity of the microbiological assay or because the data were derived from patients

under therapy for advanced colorectal or gastric carcinoma [10]. Payet et al. [14] have previously reported a mean half-life of 7.0 h; however, these authors did not distinguish between the different kinetics of l- and d-CHO-THF. Therefore, their half-life predominantly represents the slow elimination of d-CHO-THF. Our elimination half-life for CH_3-THF is within the same range as those reported by Hamel et al. [8], Newman et al. [13], Straw et al. [24, 25], and Payet et al. [14] but differs considerably from the mean value of 349 min reported by Machover et al. [10]. The elimination half-life for d-CHO-THF presented in this paper confirms the results obtained by Straw et al. [24, 25] and Rustum et al. [19].

Our data reflect the metabolism and elimination of l-folinic acid. In this investigation, approx. 60% of the body clearance of l-CHO-THF was caused by its conversion to CH_3-THF and 40%, by renal excretion. According to Straw et al. [24, 25], metabolism of l-CHO-THF predominates to an even higher degree and renal elimination comprises only 22% of the total body clearance. These differences may be explained by their lower d, l-CHO-THf doses of only 25–100 mg [24]. At higher d, l-CHO-THF doses, the metabolizing capacity of the liver may be saturated. As a consequence, a lower percentage of the total l-CHO-THF is converted to CH_3-THF and the portion that is excreted via the kidneys increases accordingly. A 3-fold increase in the d, l-CHO-THF dose from 100 mg (used by Straw et al. [24]) to 300 mg caused a 6-fold rise in the AUC for l-CHO-THF in our investigation. This supports the hypothesis that the capacity of the liver to metabolize l-CHO-THF is saturated at higher CHO-THF doses.

Due to the rapid formation of CH_3-THF and its longer half-life, we determined an AUC for CH_3-THF that was 1.5-fold higher than that of l-CHO-THF. In relation to the dose, the AUC of CH_3-THF determined in this study was on the same order as that obtained by Straw et al. [24]. Renal clearance of CH_3-THF was nearly equal to the total body clearance: without further metabolism, CH_3-THF was nearly quantitatively excreted by the kidneys.

With a terminal half-life of 438 ± 69 min, the total body and renal clearance of the biologically inactive d-CHO-THF was very slow, with values of 12.96 ml/min for total body and 12 ml/min for renal clearance. This observation suggests that d-CHO-THF is not metabolized but is exclusively excreted via the kidneys. The long d-CHO-THF half-life results in an extremely high AUC, which was in the range of 22,500 $\mu M \times$ min. The ratios of d-CHO-THF : l-CHO-THF and d-CHO-THF : CH_3-THF were high, with values of 17 and 11, respectively. Even considering both l-CHO-THF and CH_3-THF, the concentration advantage greatly favours the inactive d-stereoisomer.

The apparent volume of distribution for d-CHO-THF was considerably lower than that of l-CHO-THF and CH_3-THF. These data are in accordance with those of Straw et al. [24] and suggest that the unnatural isomer may not distribute as extensively as the naturally occurring l-folates. Indeed, Bertrand and Jolivet [1] have demonstrated the poor transport of d-CHO-THF in CCRF-CEM cells. The K_t values for d-CHO-THF were >30-fold higher than those for l-CHO-THF [1]. Thus, poor transport across cell membranes might be one reason for impaired tissue distribution.

The inter-subject variability observed in our study could have been due to the dose of 300 mg given to all individuals regardless of their body surface, the mean of which was 1.77 ± 0.16 m^2 ($\pm 9.3\%$), However, this explains only the differences in peak concentrations, which correlated inversely with the body surface [CHO-THF$_{total}$, 80.7 ± 10.2 μM (SD = 12.6%); d-CHO-THF, 53.1 ± 5.2 μM (SD = 9.8%); CH_3-THF, 5.13 ± 0.62 μM (SD = 12.2%)]. The high variation of l-CHO-THf (SD, $\pm 19.1\%$) could possibly have been so some extent by differences in its metabolism. Variability in elimination half-life, AUC and clearance is of course, more complex to explain: methodological factors (e.g., number of time points and precision of measurements) and individual factors (e.g., state of hydration and differences in liver metabolism) are involved. It also seems to be dose-related; for example, the standard deviation of the elimination half-life of folinic acid increases to >30% (l-folinic acid) and 60% (CH_3-THF) with increasing dose [23].

Concentrations of reduced folates necessary for the enhancement of 5-FU toxicity are reported to be 5 or 10 μM [3, 7]. According to Evans et al. [7], l-CHO-THF and CH_3-THF are equieffective with respect to the potentiation of thymidylate synthase inhibition by the 5-FU anabolite FdUMP. The short-term infusion of 300 mg d,l-CHO-THF resulted in concentrations of reduced l-folates (l-CHO-THF plus CH_3-THF) of >5 μM, and were thus above the threshold of 5 μM, for 2 h. 5-FU plasma clearance is characterized by a very short half-life ranging between 4.5 and 13 min; this is mainly due to its reduction to dihydrofluorouracil (FUH_2), which is then catabolized to α-fluoro-ureidopropionic acid (FUPA) and α-fluoro-β-alanine (FBAL) [16, 21]. With regard to this rapid turnover of 5-FU and to the above-discussed kinetics of l-CHO-THF and CH_3-THF, the short-term infusion of 170 mg/m^2 d,l-CHO-THF should provide sufficient concentrations of reduced l-folates to enhance FdUMP toxicity in treatment protocols where 5-FU is given as a bolus injection [6, 9, 10] or as a 2-h infusion, such as in our treatment protocols for advanced colorectal cancer.

Although transport of d-CHO-THF into cells is poor, with a K_t of 10–45 μM, d-CHO-THF acts as a competitive inhibitor of l-CHO-THF and CH_3-THF influx, with K_i values on the order of 30 μM [1]. Due to its slow elimination, d-CHO-THF can be detected at serum concentrations above the K_i of 30 μM for an extended period. Thus, it is possible that d-CHO-THF causes an impairment of cellular influx of l-CHO-THF and CH_3-THF not only in vitro but also in vivo. However, further investigations in vitro and in vivo are necessary before this question can be answered. Should this assumption prove to be true, the use of pure, biologically active l-folinic acid may result in further improvement of the efficacy of folinic acid/5-FU therapy.

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