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Accumulation of tetrahydrofolates in human plasma after leucovorin administration

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Summary. Reduced folates in plasma after i.v. and oral leucovorin administration were estimated by a ternary complex assay based on the incorporation of CH_2FH_4 into a stable complex with *Lactobacillus casei* thymidylate synthase and [^{3}H]FdUMP. Each of the reduced folates, CH_2FH_4, FH_4, and $5CH_3FH_4$, could be quantitatively recovered from plasma by this approach even in the presence of high concentrations of the parent compound leucovorin. Examination of the accumulation kinetics of these reduced folates showed that after i.v. administration of 20 mg D,L-leucovorin to a healthy volunteer, FH_4 and, to a lesser extent, CH_2FH_4 accumulated to maximal levels very early (< 15 min), with a subsequent depletion that had a half-life of approximately 30 min. Accumulation of FH_4 reached a peak level that was 12% of the maximal level of $5CH_3FH_4$ achieved and more than 3 times greater than the pretreatment level of this common, circulating reduced folate form. Similar accumulation patterns were observed in a female patient with metastatic colonic cancer who was undergoing methotrexate (MTX)/fluorouracil therapy followed by i.v. leucovorin (15 mg). FH_4 also accumulated, but to a lesser extent and over a longer period of time, when the same dose of leucovorin given orally. When several similar doses of leucovorin were given prior to the experimental dose, greater accumulation and duration of the FH_4 response was observed. We propose that accumulation of FH_4 and CH_2FH_4 could provide a circulating source of the reduced folate thought to be the active form for both high-dose MTX with leucovorin rescue and enhancement of fluorouracil activity.

Introduction

Leucovorin is currently of interest for its role as an agent in high-dose methotrexate (MTX) therapy with leucovorin rescue [2, 5, 6] as well as in the enhancement of the therapeutic effects of fluorouracil [19, 20]. Two basic approaches have previously been used to evaluate the appearance of reduced folates after either oral or i.v. leucovorin administration: (1) HPLC separation with UV identification from reference compounds [14, 23] and (2) selective microbiological growth support [8, 12]. Both approaches have shown the major component accumulating in plasma after leucovorin administration to be $5CH_3FH_4$ [12, 14]. A minor component (2%–10%) has been observed that is *Streptococcus faecalis* sensitive [12]. Since *S. faecalis* sensitivity does not distinguish among reduced folates other than $5CH_3FH_4$ [1], the specific reduced folate associated with the minor component was not determined. Chromatographic techniques can resolve individual folates, but if UV absorption detection methods are used, they lack the sensitivity to identify low levels of particularly labile reduced folates. Hence, other reduced folates in plasma following leucovorin treatment have not been thoroughly characterized. Of particular interest in terms of both MTX rescue and fluorouracil enhancement is the possible accumulation of FH_4 and CH_2FH_4, which could provide a circulating source of the thymidylate synthase substrate for direct participation in rescue and formation of the inhibitory ternary complex with the fluorouracil metabolite, FdUMP [10, 21]. Thus, a previously developed radioenzymatic assay for CH_2FH_4 and FH_4 [3, 18], based on the entrapment of limiting concentrations of CH_2FH_4 into a stable ternary complex with purified *L. casei* thymidylate synthase and tritiated FdUMP, was used to investigate the accumulation of these reduced folates in human plasma following oral and i.v. leucovorin administration.

Materials and methods

Reagents. [^{3}H]FdUMP (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif) and FH_4 was obtained from Fluka Chemical Co. (Ronkonkoma, NY). $10CHOFH_4$ was a gift from Dr. John McGuire (Rosewell Park, Buffalo, NY). $5CH_3FH_4$, $5CHOFH_4$, NADPH and all other reagents were purchased from Sigma Chemical Company (St. Louis, Mo). Thymidylate synthase (0.9 IU/mg) was purified from aminopterin-resistant *Lactobacillus casei* cells according to the method of Dunlap et al. [4] and pig liver methylenetetrahydrofolate reductase (0.25 IU/mg) was purified according to the method described by Mathews and Haywood [11].

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Abbreviations: MTX, methotrexate; FdUMP, 5-fluoro-2′-deoxyuridine-5′monophosphate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; CH_2FH_4, 5,10-methylenetetrahydrofolate; $5CH_3FH_4$, 5-methyltetrahydrofolate; $10CHOFH_4$, 10-formyltetrahydrofolate; $5CHOFH_4$, 5-formyltetrahydrofolate

Estimation of reduced folates. CH_2FH_4, FH_4 and $5CH_3FH_4$ were measured by a series of radioenzymatic assays [3, 18; Bunni et al. unpublished data] based on the entrapment of CH_2FH_4 by thymidylate synthase and [^{3}H]FdUMP to form a stable ternary complex [21]. Fresh plasma (0.5 ml) was diluted into 0.5 ml buffer solution containing 50 m*M* TRIS-HCl, 50 m*M* sodium ascorbate, 1 m*M* ethylenediaminetetraacetate (EDTA), and 0.25 *M* sucrose (pH 7.4). The diluted plasma was boiled for 3 min and centrifuged at 10,000 *g* for 10 min at 4° C. CH_2FH_4 was estimated by combining aliquots (100 µl) of diluted plasma with 16 mU thymidylate synthase and 125 n*M* [^{3}H]FdUMP (20 Ci/mmol) in a total volume of 200 µl buffer solution. Ternary complex formation was allowed to proceed at 25° C for 30 min, stopped by the addition of sodium dodecyl sulfate (SDS) (1%), and boiled for 5 min. Aliquots (25 µl) were centrifugally filtered over 400-µl minicolumns of Sephadex G-25 to separate bound from free [^{3}H]FdUMP and counted in a Packard scintillation counter to determine bound [^{3}H]FdUMP and hence CH_2FH_4, as previously described [17]. FH_4 was determined by difference in the same reaction system but in the presence of 6.5 m*M* formaldehyde to convert FH_4 to CH_2FH_4. $5CH_3FH_4$ was determined following enzymatic conversion to CH_2FH_4 using CH_2FH_4 reductase as previously described [3].

Leucovorin administration and sample collection. Leucovorin (Lederle) was obtained as a dry powder in 50-mg vials. Each vial was diluted with 5 ml sterile water prior to administration. A heparin lock was used for both i.v. leucovorin administration and blood withdrawal. Oral leucovorin was obtained as tablets (Burroughs-Welcome). Approximately 5-ml blood samples were collected in tubes containing 0.07 ml 15% EDTA to prevent coagulation and immediately centrifuged at 500 *g* for 10 min at 4° C to obtain plasma for analysis.

Subjects. A normal healthy male volunteer (90 kg) was given 20 mg leucovorin as an i.v. bolus through a heparin lock; blood samples were collected through the same lock.

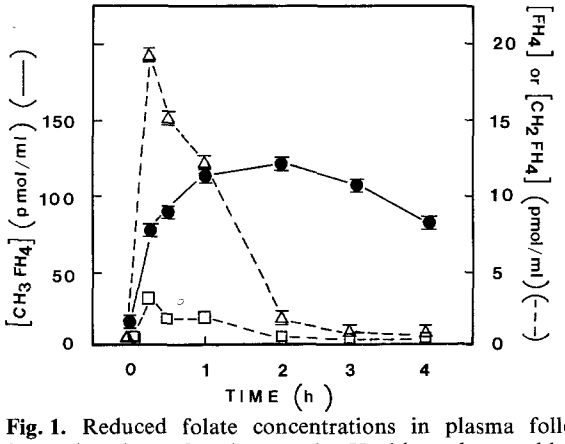

Fig. 1. Reduced folate concentrations in plasma following a 20-mg i.v. dose of D,L-leucovorin. Healthy volunteer blood samples were collected in the presence of EDTA and centrifuged immediately to obtain plasma. FH_4 (△), CH_2FH_4 (□), and $5CH_3FH_4$ (●) were assayed by incubation of 100 µl plasma with thymidylate synthase and [^{3}H]FdUMP in a total volume of 200 µl. The buffer system and other conditions are described in *Materials and methods. Points* represent the mean of four determinations and *error bars*, the range

A female volunteer patient (54 kg), undergoing therapy for metastatic colonic cancer, was given 15 mg leucovorin either orally or i.v. through a heparin lock; blood samples were collected through a separate heparin lock. At 25 h prior to the initial leucovorin dose the patient received a 300 mg infusion of MTX over 30 min, and 60 min prior to the initial i.v. leucovorin dose she received a 900-mg dose of fluorouracil. During the second treatment series, when the initial leucovorin dose was given orally the fluorouracil dose was 1200 mg. Additional 15-mg leucovorin doses were given at 6-h intervals following the first dose.

Results

Plasma concentrations of FH_4, CH_2FH_4, and $5CH_3FH_4$ were estimated by the ternary complex assay following i.v. administration of D,L-leucovorin (Fig. 1). $5CH_3FH_4$ accumulation and depletion followed a kinetic pattern very similar to that previously reported using either microbiological [12] or HPLC [23] analytical techniques. FH_4 accumulated to a peak level that was reached much earlier than that of $5CH_3FH_4$ and represents approximately 12% of the maximal accumulation of the latter. In addition, CH_2FH_4 accumulated to a lesser but significant extent, following a pattern similar to that of FH_4. The maximal accumulation of CH_2FH_4 was 63% of the pretreatment $5CH_3FH_4$ level, whereas the maximal level of FH_4 was more than 3 times the pretreatment value of this reduced folate. FH_4 was depleted with a half-life of approximately 30 min.

To verify the accuracy of the assay method used to measure the low levels of the very labile FH_4 and CH_2FH_4 pools, extensive recovery experiments were carried out. In Table 1 it can be seen that FH_4, CH_2FH_4 and $5CH_3FH_4$, added to plasma and submitted to the entire assay procedure, could be recovered at near quantitative levels. However, other reduced folates at high levels could nevertheless interfere either positively or negatively with endogenous target folate recovery, even though added reference compounds could be completely recovered. Thus, recovery experiments were conducted in which other reduced folates were added at high levels and the target folate was recovered in their presence. Of particular concern was the

Table 1. Recovery of reference reduced folates from plasma[a]

FH_4	97.6 ± 1.9
CH_2FH_4	102.7 ± 2.6
$5CH_3FH_4$	92.2 ± 2.0
FH_4 (+ $5CH_3FH_4$)	93.7 ± 4.0
FH_4 (+ $5CHOFH_4$)	94.4 ± 0.8
FH_4 (+ $10CHOFH_4$)	101.5 ± 2.5

[a] Fresh plasma (0.5 ml) from a healthy volunteer was immediately diluted in an equal volume of a buffer containing 50 m*M* TRIS-HCL, 50 m*M* sodium ascorbate, 1 m*M* EDTA, and 0.25 *M* sucrose (pH 7.4). FH_4 (20 pmol/ml), CH_2FH_4 (46 pmol/ml), and $5CH_3FH_4$ (20 pmol/ml) were added to diluted plasma prior to boiling for 3 min and centrifugation. Folates were measured by the ternary complex assay and recovery was calculated based on the anticipated sum of endogenous plus added reduced folates. On another portion of the same diluted plasma, FH_4 was added at a concentration of 20 pmol/ml and other reference reduced folates, shown in parentheses, were added at concentrations of 100 pmol/ml to test their impact on FH_4 measurements. Samples were treated as described above. Values represent the mean ± SEM for 4–6 determinations

ability of the ternary complex assay to detect FH_4 accurately in the presence of high concentrations of the parent compound, $5CHOFH_4$, and the major metabolite, $5CH_3FH_4$. Thus, these two folates were introduced at levels 5 times that of reference FH_4. Table 1 shows that even at these high levels FH_4 was recovered quantitatively. Another reduced folate that could potentially interfere and could arise from $5CHOFH_4$ is $10CHOFH_4$. Hence, this folate was also added to the FH_4 recovery system but, again, with negligible impact (see Table 1).

Furthermore, since MTX and fluorouracil, or metabolic changes resulting from their presence, could also have an impact on CH_2FH_4 and FH_4 measurements, recovery of CH_2FH_4 was examined in a plasma sample from a patient who had received these drugs. Samples collected 24 h after MTX and 1 h after fluorouracil but prior to leucovorin administration were submitted to recovery experiments in the presence of reference CH_2FH_4. In the case of MTX alone, recovery of CH_2FH_4 was 70%, whereas plasma samples taken after exposure to both MTX and fluorouracil yielded a recovery of 84%. These recoveries are somewhat lower than those obtained in the absence of drug treatment; however, they were deemed acceptable for the comparative kinetic studies that were carried out, and no correction was applied to results determined in the presence of these drugs.

The use of leucovorin as a cancer chemotherapeutic agent has two bases: enhancement of the therapeutic effects of fluorouracil [19, 20] and as a rescue agent from high-dose MTX [2, 5, 6]. In both cases it is ultimately likely to affect the target enzyme, thymidylate synthase. In MTX rescue therapy, the recovery of reduced folate pools to obtain adequate levels of the substrate, CH_2FH_4, is a potentially important mechanism. In fluorouracil enhancement, an increase in the level of the same substrate, to enhance the formation of the inhibitory ternary complex formed between thymidylate synthase, CH_2FH_4, and fluorodeoxyuridylate, could be a primary mechanism [9, 10]. Hence, the accumulation of CH_2FH_4 and FH_4 in the presence of these drugs could have an impact on their effectiveness.

To investigate the potential appearance of these reduced folates during MTX and fluorouracil therapy, their levels were examined after the administration of leucovorin to a cancer patient undergoing MTX and fluorouracil treatment. MTX (300 mg) injected i.v. over 30 min was followed 24 h later with 5-fluorouracil (900 mg for results in Figs. 2 and 3, and 1200 mg for results in Figs. 4 and 5). Leucovorin (15 mg) was given 1 h later, and additional doses were given thereafter at 6-h intervals for 2 days. Figure 2 shows the accumulation of FH_4, CH_2FH_4, and $5CH_3FH_4$ subsequent to the first leucovorin dose, which was given as an i.v. bolus (15 mg in 1.5 ml sterile water). The accumulation patterns in this patient were similar to those in the healthy volunteer. FH_4 reached a maximal level within 15 min; this level was 10% of the maximal level of $5CH_3FH_4$ and 7.6 times higher than the pretreatment level of $5CH_3FH_4$. CH_2FH_4 accumulated at low but readily detectable levels. Analysis of plasma samples just prior to and immediately following MTX and fluorouracil treatment showed no detectable FH_4 or CH_2FH_4, and $5CH_3FH_4$ levels were unchanged by the drugs. However, $5CH_3FH_4$ was elevated considerably in plasma just prior to the *fourth* leucovorin dose (Fig. 3), presumably as a result of prior

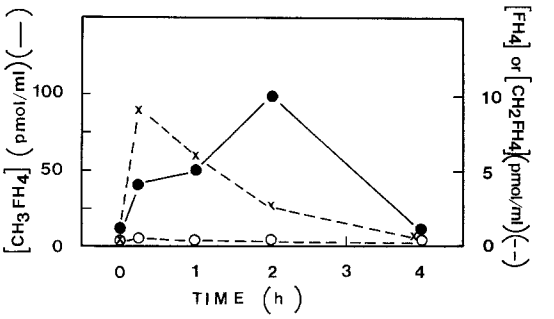

Fig. 2. Reduced folate concentrations in plasma after i.v. administration of leucovorin to a colonic cancer patient undergoing treatment. Blood samples were collected before and following a 15-mg leucovorin dose given 25 h after MTX (300 mg) and 1 h after fluorouracil (900 mg). Plasma was assayed for FH_4 (X), CH_2FH_4 (O), and $5CH_3FH_4$ (●) as described in Fig. 1

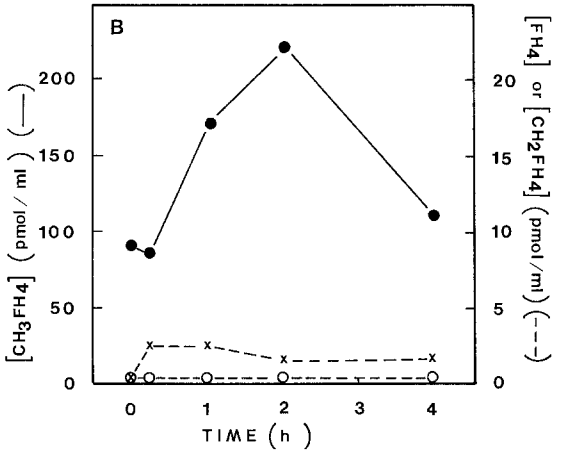

Fig. 3. Reduced folate concentrations in plasma following an oral leucovorin dose given during the same treatment as in Fig. 2, except that samples were collected just before and following the last of three 15-mg leucovorin doses given orally at 6-h intervals after the initial i.v. dose (15 mg). Plasma samples were collected and assayed for FH_4 (X), CH_2FH_4 (O), and $5CH_3FH_4$ (●) as described in Fig. 1

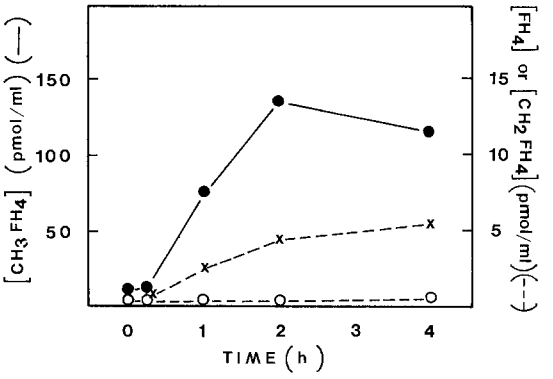

Fig. 4. Reduced folate concentrations in plasma after an oral leucovorin dose (15 mg) given 1 h after MTX/fluorouracil treatment. Drug treatment was the same as in Figs. 2 and 3 except that the fluorouracil dose was 1200 mg. Plasma samples were assayed for FH_4 (X), CH_2FH_4 (O), and $5CH_3FH_4$ (●) as described in Fig. 1

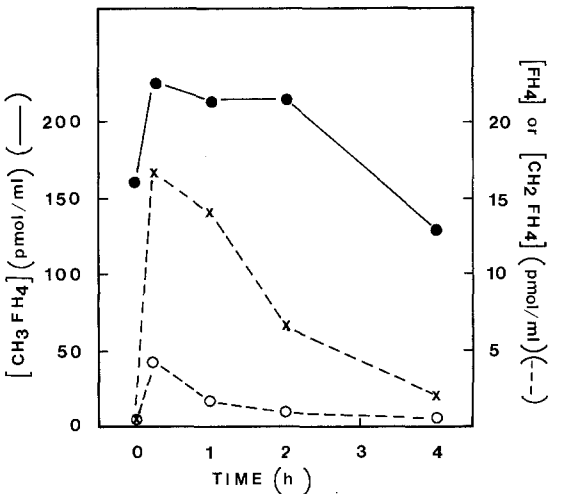

Fig. 5. Reduced folate concentrations in plasma after an i.v. dose (15 mg) of leucovorin given in the same series as Fig. 4, except that the patient had three prior oral leucovorin doses at 6-h intervals. Blood samples were collected and assayed for FH_4 (X), CH_2FH_4 (O), and $5CH_3FH_4$ (●) as described in Fig. 1

doses. Each dose subsequent to the first was given orally as a 15-mg tablet. $5CH_3FH_4$ followed a kinetic pattern that was somewhat delayed but generally similar to that observed when the initial leucovorin dose was given i.v.; however, it attained a maximal level approximately twice that achieved with the initial i.v. dose (see Fig. 2). FH_4 did not attain as high a level as that obtained with initial i.v. administration, but the duration of response was considerably longer. CH_2FH_4 was again recorded at low but detectable levels.

When the same patient underwent a second treatment regime at a later date, the initial leucovorin dose was given orally (Fig. 4) and the fourth was injected i.v. (Fig. 5), with intervening doses given orally. Figure 4 shows that initial oral administration resulted in a delayed accumulation of all reduced folates as compared with that resulting from initial i.v. administration (see Fig. 2). Furthermore, FH_4 and CH_2FH_4 increased nearly linearily over the 4-h experiment without evidence of the early maximum observed in the other case. When an i.v. dose was given after three prior oral doses (Fig. 5), FH_4 and CH_2FH_4 achieved the early maxima and also achieved significantly greater levels than when leucovorin was injected i.v. without prior oral doses (see Fig. 2). $5CH_3FH_4$ may have followed a somewhat different kinetic course in this case, but maximal levels reached were generally the same as in Fig. 3. Hence, this condition gave rise to the greatest peak accumulation and overall sustained level of FH_4 and CH_2FH_4 of any of the conditions used, with little difference in the total accumulation of $5CH_3FH_4$.

Discussion

Investigation of labile, low-level reduced folates in plasma after leucovorin administration has been difficult by classical analytical techniques. The extremely sensitive microbiological approach has been used to observe low levels of reduced folates other than $5CH_3FH_4$ after leucovorin administration, but it could not distinguish the specific reduced folates present [12]. More direct HPLC techniques have been used to show the accumulation of $5CH_3FH_4$ and the parent compound $5CHOFH_4$ [14, 23], but more labile, minor constituents have not readily been observed by this approach. Nixon and Bertino [13] have observed small amounts of material that chromatographed as either $10CHOFH_4$ or 5,10-methenyltetrahydrofolate 75 min after the administration of a dose of radiolabeled leucovorin. On the other hand, Perry and Chanarin [15] have suggested that the small amount of *S. faecalis*-sensitive activity observed following oral doses was due to folic acid. Pratt and Cooper [16] and Whitehead et al. [24] have suggested that the *S. faecalis*-sensitive activity was due to the parent compound, $5CHOFH_4$. Hence, there does not appear to be complete agreement as to the identity of minor components, although their presence has clearly been shown.

The use of the ternary complex assay for the identification of minor components has overcome some of the analytical difficulties involving the very labile FH_4 and CH_2FH_4 pools. Recoveries of these pools from plasma can be seen to be essentially quantitative (Table 1). Although the recovery of CH_2FH_4 from the plasma of an MTX- and fluorouracil-treated patient was somewhat diminished [7, 22], it was deemed suitable for the present study without correction. Examination of $5CH_3FH_4$ with this methodology showed that the results obtained were very similar to those observed by others using microbiological and chromatographic approaches. Furthermore, the major reduced folate pools in plasma following leucovorin administration are the parent compound and $5CH_3FH_4$, neither of which significantly influenced the estimation of FH_4. Moreover, $10CHOFH_4$, which is a potential metabolite of leuocovorin, also did not interfere with the assay methodology.

It has previously been shown that leucovorin, when administered orally vs i.v., gives rise to somewhat different accumulation patterns for both the major and minor metabolites in plasma [12]. Nevertheless, administration by both routes results in selective use of L-leucovorin, with accumulation of $5CH_3FH_4$ as the predominant metabolite [23]. After i.v. administration, L-leucovorin reportedly [23] disappears from the circulatory system, with a half-life of approximately 32 min. After an initial accumulation FH_4 was seen to be depleted, with a similar half-life (approximately 30 min), whereas $5CH_3FH_4$ survives much longer, with a half-life of 224 min [23]. It is not clear whether FH_4 depletion results from instability, metabolism, or tissue uptake; however, should a significant proportion of this depletion result from uptake, support for the concept of a more readily available source of CH_2FH_4 would be enhanced.

Although the relative accumulation of FH_4 is not quantitatively as great as that of $5CH_3FH_4$, levels achieved even at the relatively low leucovorin doses given in these experiments were significant. With i.v. administration, levels as great as 12% of the maximal level of $5CH_3FH_4$ were observed; this level is several times greater than typical circulating levels of $5CH_3FH_4$. Accumulation following oral administration follows a somewhat delayed pattern as compared with that obtained after i.v. injection, and levels attained were not as great. The levels achieved were nevertheless much greater than the pretreatment levels of the predominant circulating reduced folate. Furthermore, the leucovorin doses used are typical of those used for MTX therapy, but in the case of fluorouracil enhancement therapy, doses as much as 25 times greater than these are often

given [20], which would be expected to result in even greater accumulation of FH_4.

McGuire et al. [12] found that increased oral doses of leucovorin resulted in a saturation pattern for the accumulation of $5CH_3FH_4$ (estimated from *L. casei* activity). On the other hand, the *S. faecalis* activity increased linearly with dose up to the highest level used (200 mg). Since *S. faecalis* activity does not distinguish between reduced folates other than $5CH_3FH_4$, these authors suggested that this nonsaturable component could be unmetabolized parent compound, other formylated folates, or FH_4. If FH_4 as observed in the present work represents a prominent portion of the *S. faecalis* activity previously observed, then the level achievable at high therapeutic levels could be much greater than that seen at the 15- to 20-mg doses used here.

The mechanism by which FH_4 accumulates in plasma after leucovorin administration remains unclear. The difference in the accumulation pattern for FH_4 between i.v. and oral administration could be the result of differences in metabolic sites; however, other possibilities also exist. Future work will address the underlying reasons for administration route differences and attempt to identify the metabolic origin of the plasma FH_4.

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