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Plasma kinetic study of folinic acid and 5-methyltetrahydrofolate in healthy volunteers and cancer patients by high-performance liquid chromatography*

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Summary. A reversed-phase HPLC method is described for the simultaneous determination of folinic acid, MTX, and their plasma metabolites 5-CH_3-FH_4 and 7-OH-MTX respectively. In addition, this technique allows the separation of FA another naturally occurring folate, and of AMT, used as internal standard.

Separation of these compounds was achieved on a Waters Spherical C_{18} column at a flow rate of 0.8 ml.min^{-1}. Elution was carried out with 0.1 *M* sodium acetate buffer (pH 5.5) as solvent A and 7.5% acetonitrile 92.5% bidistilled water as solvent B. UV detection was performed at 280 nm. This method was applied in a pharmacokinetic study of folinic acid and its plasma metabolite 5-CH_3-FH_4 following two different protocols: (1) i. v. bolus injection of 50 mg calcium folinate in six healthy volunteers and (2) simultaneous i. v. bolus injections of 50 mg/m^2 MTX and 50 mg/m^2 folinic acid in four cancer patients. Mean apparent half-life values for folinic acid and its metabolite were 7.02 ± 1.81 h and 3.90 ± 0.86 respectively in the first protocol, 4.80 ± 1.48 h and 4.74 ± 1.47 h in the second protocol. MTX and 7-OH-MTX were also quantified in the second protocol and were found not to affect the pharmacokinetics of folinic acid and 5-CH_3-FH_4.

Since in vitro studies on metabolism of folinic acid might be of great interest in trying to assess the mechanism of action of the folates and the potential interaction of MTX and 7-OH-MTX in this mechanism via the metabolism, the chromatographic method we describe here has been adapted for the separation of all the potential intracellular monoglutamyl metabolites of folinic acid.

Introduction

Folinic acid has widespread clinical application, especially in folate deficiency and in chemotherapy based on middle- or high-dose MTX [16] and 5-fluorouracil [45] therapies.

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Abbreviations: FH_2, dihydrofolate; FH_4, tetrahydrofolate; 5-CHO-FH_4, 5-formyltetrahydrofolate; 10-CHO-FH_4, 10-formyltetrahydrofolate; 5-CH_3-FH_4, 5-methyltetrahydrofolate; 5,10-CH=FH_4, 5,10-methenyltetrahydrofolate; MTX, methotrexate; 7-OH-MTX, 7-hydroxymethotrexate; AMT, aminopterin; HPLC, high-performance liquid chromatography

The optimization of folinic acid regimens requires an understanding of the disposition of this agent. This can be achieved by the characterization of its pharmacokinetics in biological fluids, but also by its metabolism in cellular models. The identification of naturally occurring folates is, indeed, complicated by the large number of possible derivatives arising from different combinations of one-carbon moieties, oxidation states, and glutamyl chain lengths. It is now well established that these various folate forms differ in their biological activity [6].

Investigations of the mechanisms by which folinic acid induces the regulation of the movement of one-carbon units through these pathways after high-dose MTX therapy have been hampered by the lack of available pharmacokinetic data for both folinic acid and 5-CH_3-FH_4. This was due partly to the inavailability of technologies appropriate for simultaneous evaluation of the individual folate forms. Although the various forms may be separated and quantitated by conventional ion-exchange chromatography on derivatized cellulose, these procedures suffer from a lack of sensitivity and are time-consuming. Recently, rapid separation of folate derivatives has been accomplished using anion-exchange HPLC [5, 13–15, 26, 27, 31, 33, 40, 41, 48] or paired-ion reversed-phase HPLC (2, 8, 10, 24, 28, 36, 42, 43]. Most of these methods allow the resolution of only few natural folate forms and do not permit the simultaneous determination of folates and antifolates. Moreover, they depend on microbiological assays or radioactivity to quantify the drugs of interest.

In this report we describe an improved HPLC method suitable for the simultaneous plasma quantification of individual folate forms, MTX and its hydroxylated metabolite after i. v. administration of folinic acid and/or MTX.

This method was then used to compare plasma kinetics of folinic acid and 5-CH_3-FH_4 in six healthy volunteers receiving 50 mg calcium folinate and four patients receiving 50 mg/m^2 each of MTX and calcium folinate.

Materials and methods

Reagents. Folic acid, FH_2, FH_4, 5-CH_3-FH_4, and 2-mercaptoethanol were purchased from Sigma (St Louis, Mo., USA). MTX, AMT, and folinic acid were kindly supplied by Lederle Laboratories (Oullins, France). 7-OH-MTX was synthetized in our laboratory as reported previously [19]. MTX and 7-OH-MTX were purified on DEAE cellulose [21]. A mixture of 5,10-CH-FH_4 and 10-CHO-FH_4 was

generated by isomerization of folinic acid, first at pH 1.5, then at pH 6.0 [34]. All solutions of standard folates were protected from light and stored at −20 °C in 0.2 *M* 2-mercaptoethanol to prevent oxidative degradation.

HPLC assay. HPLC analyses were performed with a Hewlett-Packard HP 1090 equipped with a Rheodyne fixed-loop injector of 100 µl, a calculator-integrator HP 3390 A, and a filter-photometric detector at 280 nm. The temperature of the column was adjusted to 40 °C. The column was a Waters Spherical C_{18} (150 × 4.6 mm; particle size 5 µm) protected by a Rheodyne column inlet filter (pore size 2 µm). Elution was carried out at a flow rate of 0.8 ml.min^{-1} with 0.1 *M* sodium acetate buffer (pH 5.5) as solvent A and 7.5% acetonitrile 92.5% bidistilled water as solvent B. Solvent B gradient elution characteristics for folates and antifolates separation are reported below under "Results".

Clinical regimens of CF administration. This study included two folinic acid administration protocols:

The first protocol included a kinetic study of folinic acid and 5-CH_3-FH_4 after i. v. administration of 50 mg calcium folinate to six healthy volunteers. Calcium folinate (Lederfoline) is provided as a 50-mg dry residue to be dissolved in 10 ml of 5% dextrose and injected as an i. v. bolus.

The second protocol included a simultaneous kinetic study of folinic acid, MTX, and their respective plasma metabolites 5-CH_3-FH_4 and 7-OH-MTX after i. v. administration of 50 mg/m^2 calcium folinate and MTX to four patients. This latter protocol permits an identification of the kinetic parameters of antifolate before high-dose MTX administration to patients (2 g/m^2).

The volunteers' and patients' characteristics and clinical information are given in Table 1.

Sample collection protocol. In the first protocol, blood samples were collected before drug administration and at the following times after the i. v. bolus: 10, 20, 30, and 45 min, 1, 1.5, 2, 2.5, 3, 5, 8, and 12 h. In the second protocol, they were collected before administration of both MTX and calcium folinate, and at the following times after the i. v. bolus: 15, 30, and 45 min, 1, 3, 4, 6, and 12 h.

Sample stability and extraction procedure. Stability studies were undertaken particularly on 5-CH_3-FH_4 since this compound had been reported to be rapidly oxidized. At room temperature, 5-CH_3-FH_4 degradation occurred very rapidly (half-life = 1.0 h). However, in the presence of both sodium ascorbate and 2-mercaptoethanol at −20 °C, the oxidative degradation of 5-CH_3-FH_4 was less than 10% after 2 days. The stabilities of folinic acid, MTX, and 7-OH-MTX were also investigated in plasma obtained from untreated patients. No degradation was observed over 3 months for any of these compounds stored at −20 °C.

Blood samples were collected in heparinized tubes containing 2 mg sodium ascorbate/ml blood. After plasma separation, 2-mercaptoethanol (0.2 *M* final concentration) was immediately added to the sample in order to inhibit the oxidative degradation. The internal standard (10 µl = 1 nmol AMT) was added to 180 µl heparinized plasma containing 0.2 *M* 2-mercaptoethanol. Proteins were precipitated by adding 10 µl 50% trichloroacetic acid and pelleted by centrifugation (15000 *g*, 10 min, 4 °C). Supernatant fluid (100 µl) was injected directly onto the column.

Parameter determination. C_{max} and T_{max} were graphically evaluated. The time-concentration curves were extrapolated to infinite time, $T_{1/2}$ was calculated by the determination of its slope, α ($T_{1/2} = \ln 2/\alpha$, and $AUC_{0\to\infty}$ according to the trapezoidal rule.

Results

HPLC characteristics

The resolution of folinic acid from 5-CH_3-FH_4 [4], a natural endogenous folate, MTX and 7-OH-MTX, was performed using a 8-min linear gradient from 4% to 12% of solvent B followed by a 10-min linear gradient from 12% to 65% of solvent B. These conditions allowed to resolve also AMT which was chosen as the internal standard since it is structurally similar to MTX [19]. It can be seen in Fig. 1 that all compounds were baseline-separated. The identity of each peak was confirmed by injecting each derivative separately.

Although these chromatographic conditions were similar to those previously described to resolve antifolates and their metabolites, earlier methods were never adapted to the simultaneous quantification of folates and antifolates in the presence of an internal standard. Calibration curves

Table 1. Characteristics and clinical information concerning the subjects included in the two protocols of folinic acid administration

No.	Age	Sex	Weight (kg)	Administered Dose (mg)		Diagnostic
				CF	MTX	
1	29	M	65	50	–	Healthy volunteer
2	27	M	78	50	–	Healthy volunteer
3	26	M	63	50	–	Healthy volunteer
4	25	M	63	50	–	Healthy volunteer
5	25	M	62	50	–	Healthy volunteer
6	21	M	76	50	–	Healthy volunteer
7	49	F	43	68	68	Immunoblastic lymphoma
8	70	F	65	80	80	Metastatic adenocarcinoma
9	39	F	50	75	75	Large cell malignant lymphoma
10	37	F	43	80	80	Adenocarcinoma

Numbers 1 to 6: Healthy volunteers receiving 50 mg of calcium folinate (CF) alone. Numbers 7 to 10: patients receiving simultaneously 50 mg/m^2 each of calcium folinate (CF) and methotrexate (MTX)

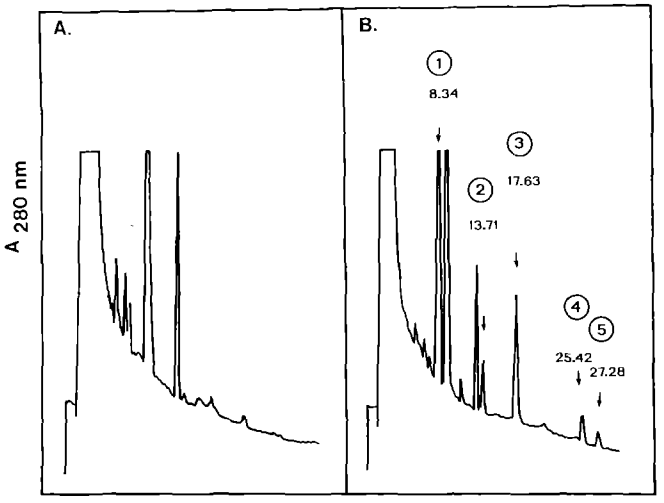

Fig. 1 A, B. Chromatograms of **A** blank plasma of patient and **B** plasma of treated patients 4 h after simultaneous i. v. administration of folinic acid and MTX (50 mg/m^2). Peaks are labeled as follows: *1*, folinic acid; *2*, 5-CH_3FH_4; *3*, AMT; *4*, MTX; *5*, 7-OH-MTX

were obtained using blank plasma spiked with folinic acid, MTX, 7-OH-MTX, 5-CH_3-FH_4, and AMT as internal standard. The ratio of analyzed compound to internal standard peak area, calculated as a function of analyzed compound concentration, was linear over the therapeutic concentration range tested (0.1–2.5 nmol injected; $n=6$). Characteristics of the calibration curves are reported in Table 2 and were routinely performed on three points. The sensitivity of the method, defined as twice the value of the signal-to-noise ratio, was approximatively 5 pmol for each of these compounds. The reproducibility of the method was evaluated at 1 and 10 nmol/ml for the different compounds added to blank plasma and according to the sample clean-up procedure and chromatographic conditions as described in "Materials and methods". The relative standard deviation for repeated analyses ($n=10$) of the same sample (within batch) or of different samples (between batches) were determined (Table 3). The possible interference in the chromatographic determination of the compounds of interest by a number of drugs, especially anticancer agents (doxorubicin, cisplatinum, trimethoprim, fluorouracil, dacarbazine, vindesine, vincristine,

Table 2. Quantitative determination of folinic acid, 5-methyltetrahydrofolate (5-CH_3-FH_4), methotrexate (MTX), and 7-hydroxymethotrexate (7-OH-MTX) according to the HPLC method described in the text

Compound	Linear regression equation[a]	Regression coefficient	Range of linearity[b]
folinic acid	Y = 2954.453 X − 89.706	0.9997	0.1–2.5
5-CH_3-FH_4	Y = 3330.023 X + 44.463	0.9996	0.1–2.5
MTX	Y = 1553.613 X + 4.304	0.9997	0.1–2.5
7-OH-MTX	Y = 1097.308 X + 54.152	0.9989	0.1–2.5

[a] Y, area counts/1000 as reported on Hewlett-Packard Model 3390 A integrator; X, nanomoles of compound injected onto the column

[b] Linearity of the response was studied between 0.1 and 2.5 nmol at the following amounts injected: 0.10, 0.25, 0.50, 0.75, 1.00, and 2.50 nmol

Table 3. Reproducibility of the HPLC determinations of folinic acid, 5-methyltetrahydrofolate (5-CH_3-FH_4), methotrexate (MTX), and 7-hydroxymethotrexate (7-OH-MTX) for repeated analyses of the same sample (within batch, $n = 10$) and of different samples (between batches, $n = 10$) in extracted plasma samples spiked with standards

Compound	Amount injected onto column (nmol)	Coeffcient of Variation	
		Within batch (%)	Between batches (%)
folinic acid	0.1	4.34	9.36
	1.0	0.24	4.73
5-CH_3-FH_4	0.1	7.61	7.84
	1.0	4.23	5.12
MTX	0.1	5.01	2.75
	1.0	0.27	5.61
7-OH-MTX	0.1	8.28	3.82
	1.0	1.47	3.48

etoposide, and teniposide) and other frequently coprescribed drugs (sulfamethoxazole, metopimazine, netilmicine, ampicillin, and cefatoline), was examined by addition of these drugs to plasma. At the detection wavelength used, only dacarbazine interfered with 5-CH_3-FH_4. This drug was never included in the treatment protocol of these patients.

Clinical applications

Mean plasma profiles for folinic acid and 5-CH_3-FH_4 after i. v. administration of 50 mg calcium folinate to healthy volunteers are illustrated in Fig. 2. Plasma level of folinic acid decreased very rapidly during the first hour and much more slowly during the remaining period of observation

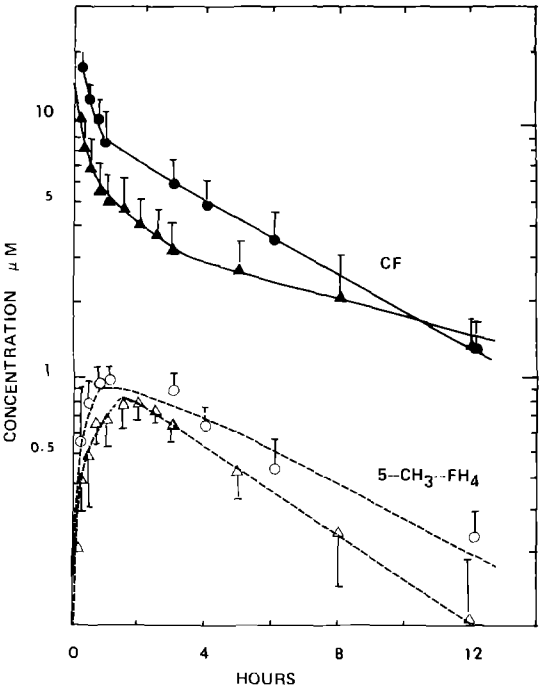

Fig. 2. Mean plasma concentrations of folinic acid *(closed symbols)* and 5-CH_3-FH_4 *(open symbols)* after i. v. bolus injection of 50 mg calcium folinate to six healthy volunteers *(triangles)* or 50 mg/m^2 folinic acid and 50 mg/m^2 MTX to four patients *(circles)*

Table 4. Mean plasma kinetic parameters of folinic acid, 5-methyltetrahydrofolate (5-CH_3-FH_4, methotrexate (MTX), and 7-hydroxymethotrexate (7-OH-MTX) after administration in healthy volunteers or patients according to the drug administration protocols described in the text

Kinetics parameters	Healthy volunteers ($n = 6$)	Patients ($n = 4$)
Folinic acid		
AUC (μmol/l/h)	100.11 ± 28.50	131.22 ± 20.41
CL (l/h)	2.07 ± 0.62	2.27 ± 0.47
$t_{1/2}$ (h)	7.00 ± 1.08	4.80 ± 1.50
5-CH_3-FH_4		
AUC	11.87 ± 1.81	15.13 ± 6.57
$t_{1/2}$	3.90 ± 0.9	4.70 ± 1.50
C_{max} (μ*M*)	0.78 ± 0.12	0.88 ± 0.26
T_{max} (h)	2.10 ± 0.4	1.40 ± 1.00
MTX		
AUC	–	21.09 ± 13.09
Cl	–	7.75 ± 2.34
$t_{1/2}$	–	2.20 ± 0.70
7-OH-MTX		
AUC	–	5.60 ± 3.80
$t_{1/2}$	–	10.70 ± 4.10
C_{max}	–	1.09 ± 0.78
T_{max}	–	4.70 ± 1.51

(mean terminal half-life value = 7.02 ± 1.81 h). Folinic acid was significantly metabolized to its 5-methylated derivative. At the earliest collection time, 10 min, the metabolite was already present in the plasma. Its level increased during the first hour, reached its C_{max} after a short period, and then decreased with a mean half-life value of 3.90 ± 0.86 h (n=6; Table 4). The mean kinetic parameters T_{max} and C_{max} for 5-CH_3-FH_4 were 2.08 ± 0.38 h and 0.78 ± 0.12 μ*M* respectively (Table 4).

Figure 2 also illustrates the mean plasma profiles for folinic acid and its methylated derivative after a simultaneous i. v. injection of calcium folinate and MTX (50 mg/m^2) to four patients. Plasma kinetics for folinic acid and its metabolite were similar to those obtained in healthy volunteers, with T_{max} and C_{max} for the metabolite of 1.44 ± 1.05 h and 0.88 ± 0.29 μ*M* respectively (Table 4). Mean half-life values were 4.74 ± 1.47 h for the metabolite and 4.80 ± 1.48 h for folinic acid (Table 4). These values were not significantly different from those obtained in the volunteers.

The total plasmatic clearance of folinic acid was 2.07 ± 0.62 l/h when administered alone, and 2.27 ± 0.47 l/h when associated with MTX in a simultaneous i. v. bolus injection.

The AUC ratios of methylated derivative under the parent folate were 0.12 ± 0.02 and 0.12 ± 0.08 for the first and second protocol respectively.

Plasma kinetics for both MTX and 7-OH-MTX are illustrated in Fig. 4. MTX plasma disappearance was biphasic, with a very rapid first phase and a slower second phase and an apparent terminal half-life value of 2.30 ± 0.44 h (Table 4). 7-OH-MTX was not detectable in plasma until the second to third hour of observation. Then its level increased to achieve a C_{max} value of 1.09 ± 0.78 μM after 4.75 ± 1.50 h (T_{max}). In all cases, the plasma terminal half-life for the metabolite was longer than that observed for the unchanged compound. The longer terminal half-life value obtained for 7-OH-MTX compared to MTX was in agreement with previously reported observations [7, 9, 18, 32]. However, the differences observed between the half-life values reported in this study for MTX and 7-OH-MTX, and those reported elsewhere, are a consequence of the determination period for this parameter. In other studies, terminal half-life values were determined on the first and/or the second day following MTX administration,

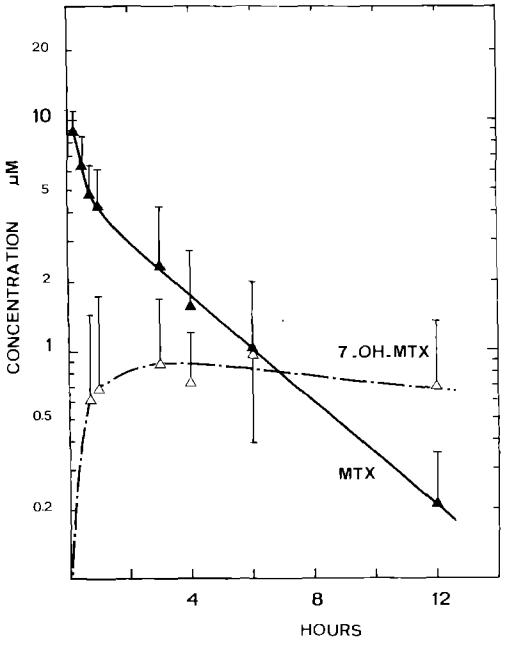

Fig. 3. Mean plasma concentrations of MTX (△———△) and 7-OH-MTX (▲———▲) after i. v. bolus injection of 50 mg/m^2 MTX to four patients

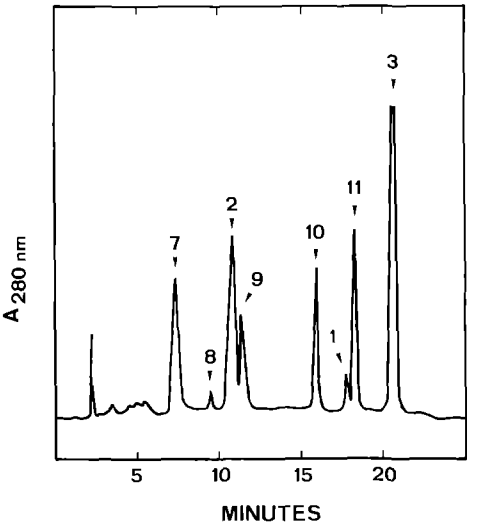

Fig. 4. Chromatogram of folinic acid and its potential intracellular derivatives. Chromatographic conditions are described under "Results". Peaks are labeled as follows: *1*, folic acid; *2*, folinic acid; *3*, 5-CH_3-FH_4; *7*, 10-CHO-FH_4; *8*, FH_4, *9*, 2-mercaptoethanol; *10*, 5,10-CH=FH_4; *11*, FH_2

while in this report the apparent terminal half-life values were estimated during a shorter period of time.

Discussion

Goldin and al. [22] first demonstrated that delayed administration of folinic acid could protect normal mice from toxic doses of MTX and could prevent most MTX-induced toxicity without decreasing the antitumor activity of the drug. Since these observations, high-dose MTX associated with folinic acid rescue has been used in the treatment of several human tumors.

The mechanism by which folinic acid enhances the therapeutic index of MTX is not well established [1, 37]. The optimization of folinic acid regimens necessitates the characterization of its pharmacokinetics and its disposition in both biological fluids and the cellular compartment. Several methods based on HPLC, using anion-exchange or paired-ion reversed-phase methods, have been reported for the quantification of different forms of folates. Few of them allow a good resolution of folate forms, and some of them require radiolabeled standards for a quantitative determination. Previously described methods for folinic acid quantification include microbiological assay [35], radiochemical assay [44], radioimmunology [30], and spectrophotometry coupled [5] or not [36] with amperometric detection. Only the method described by Montgomery et al. [36] allows the simultaneous and accurate quantification of folinic acid and 5-CH_3-FH_4 in biological fluids. The approach we use here to separate folinic acid from other naturally occurring folates and antifolates is a modification of the method described by Cashmore et al. [11]. This technique was then adapted by Kamen et al. [29] for the resolution of MTX from its impurities, and by Fabre et al. [20] for the resolution of 7-OH-MTX from its polyglutamyl derivatives. We have also employed this method, using the same solvents and a C_{18} analytical column, but the compounds are eluted by a solvent B gradient profile different than those proposed previously by these authors.

The plasmatic pharmacokinetic study of the distribution of folates is of a greater interest. Although clinically used calcium folinate is a racemic mixture of both *l*- and *d*-isomers of folinic acid, only the *l*-isomer is suspected to have pharmacological activity. Straw et al. [50], using the HPLC method described by Montgomery et al. [36] coupled to a microbiological assay, selectively quantified the *l*- and *d*-isomers of folinic acid and its 5-methylated derivative. They demonstrated the important in vivo metabolism of *l*-folinic acid to 5-CH_3-FH_4. Whichever route choosen for folinic acid administration (i. m., p. o. or i. v.), it is intensively and quickly converted to 5-CH_3-FH_4 and it is now well established that only the *l*-isomer of folinic acid would participate in this metabolic conversion [3, 47, 49, 50]. The two main sites for this biotransformation are the small intestine (jejunum) and the liver, although there is disagreement on the importance of the hepatic contribution [38, 50]. Chanarin and al. [12] explained the appearance of plasmatic 5-CH_3-FH_4 after parenteral administration of folinic acid by a fast and complete exchange of reduced folate compound with tissue 5-CH_3-FH_4 (storage).

The metabolism of folinic acid after simultaneous i. v. administration of 50 mg/m^2 each of calcium folinate and MTX does not seem to be altered by the presence of the antifolate. C_{max} and AUC of the plasmatic methylated derivative are not significantly different from those observed after i. v. administration of folinic acid alone to healthy volunteers. The apparent terminal half-live of elimination for 5-CH_3-FH_4 and folinic acid remain relatively constant in the absence or presence of MTX, and the clearance of the unchanged compound is not affected by the presence of the antifolate (at the dose of 50 mg/m^2).

The plasmatic repartition of the circulating folates (*l*- and *d*-folinic acid and 5-CH_3-FH_4) is important. Indeed, several questions remain concerning stereospecificity between the two isomers and the selectivity of formylated or methylated folate derivatives for the rescue of different normal and malignant cell lines. Sirotnak et al. [47] have shown in in vitro studies that *l*-folinic acid was a better substrate than the *d*-isomer for the reduced folate-MTX transport carrier. Halpern and al. [25] and later Dudman et al. [17] demonstrated, in tissue culture and in lymphoblast cell lines, that 5-CH_3-FH_4 protected safe tissue from MTX cytotoxicity (0.04 μM), but not the malignant tissue, since these malignant cells were characterized by a deficiency of vitamin B12-dependent-N^5-methyltetrahydrofolate-methyl transferase. This selectivity in tissue protection was not observed for folinic acid. Controversially, Groff et al. [23], using human lymphoid cell lines of normal or neoplastic origin in the presence of higher MTX concentrations than those used by Dudman et al. [17] and Halpern et al. [25], have observed that neither folinic acid nor 5-CH_3-FH_4 show any selectivity in tissue protection.

According to Pinedo et al. [39], the ratio of plasmatic concentration of folinic acid vs MTX is important in the rescue: when the MTX level is low, better therapeutic selective effectiveness of folinic acid treatment will occur. Thus, folinic acid rescue during a high-dose MTX regimen implies that it is desirable to control the metabolic profile of this folate, and to establish the best folinic acid administration schedule in order to get a favorable 5-CH_3-FH_4/folinic acid ratio. This ratio is around 0.12 when folinic acid is administered alone via the i. v. route and is not altered in the presence of MTX. This low value compared with that observed for oral administration of folinic acid alone (data not shown) is due to the important level of unmetabolized folinic acid (probably *d*-folinic acid) in a parenteral administration. Straw et al. [50] have shown that the *d*-isomer quickly becomes the major plasmatic isomer after i. v. folinic acid administration; thus, the apparent terminal half-life values we have observed for this molecule would be due to the slow plasmatic disappearance of the *d*-isomer, which is slowly accumulated in the cells. It is possible that high concentrations of the *d*-isomer interfere with the intracellular accumulation of the active form of folate for the rescue of normal cells, and so intereact, with folates at the intracellular metabolic enzyme system level, such as folylpolyglutamate synthetase which has a better affinity for the *d*-isomer [46].

The complex mechanism by which folinic acid saves normal tissues from MTX toxicity without affecting the toxicity on malignant cells remains obscure, and its assessment would be helpful for an efficient pharmacokinetic interpretation.

The HPLC method described in this paper will also permit a cellular approach to the metabolism of folinic acid, since it allows the baseline separation of folinic acid from its major intracellular monoglutamate metabolites.

Figure 4 illustrates the elution profile obtained when 10-CHO-FH_4, FH_4, folinic acid, 5,10-CH=FH_4, folic acid, FH_2, and 5-CH_3-FH_4 were analyzed simultaneously. Under these conditions, a good resolution of all these compounds was achieved except for FH_2 and folic acid. Cellular kinetic and metabolic studies of folinic acid are currently in progress.

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