EFFECTIVE ABSORPTION AND UTILIZATION OF ORAL FORMYLTHREONINE IN MAN

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Abstract Reversal of the effects of antifolates requires effective expansion of the body pool of reduced folate coenzymes. In the past, this has required parenteral use of 5-formyltetrahydrofolate. By use of radiolabeled 5-formyltetrahydrofolate, we showed that its oral administration also expands the body reduced folate pool. After oral administration of 5-formyl-14C-tetrahydrofolate-3H to fasting subjects, the labels appeared in the serum, peaking at 60 minutes. Chromatographic analysis showed that the labeled serum folate was principally the naturally occurring 5-methyltetrahydrofolate-3H. Close to 90 per cent of orally administered 5-formyltetrahydrofolate appeared to be absorbed. The most constant labeled urinary folate was found to be 10-formyltetrahydrofolate or 5, 10-methylenetetrahydrofolate. Renal excretion of labeled 5-formyltetrahydrofolate occurred at undetectable serum levels whereas renal excretion of labeled 5-methyltetrahydrofolate was proportional to its serum concentration.

Of the reduced folates, the one that is chemically most stable and is therefore used therapeutically to reverse the effects of folate antagonists, such as methotrexate, is 5-formyltetrahydrofolate (leucovorin, citrovorum factor, 5-formylFH4). However, in vitro, under acid conditions such as exist in the stomach, 5-formylFH4 isomerizes to the stable 5, 10-methylenylFH4, which, under neutral pH conditions such as exist in the jejunum, isomerizes in turn to the readily oxidized and unstable 10-formylFH4. Therefore, in the past, 5-formylFH4 has always been administered parenterally and, indeed, its oral absorption has only recently been the subject of investigations.

Increasing experience with, and use of, cancer chemotherapeutic agents suggests a place for an effective orally administered reduced folate for reversal of the effects of methotrexate. When 5-formylFH4 was administered 24 or 36 hours after methotrexate, very high doses of the folate antagonists were safely administered to patients with acute lymphatic leukemia or epidermoid carcinoma of the head and neck, with apparent improvement of the therapeutic index. The basis for this differential rescue is not clear but may depend upon the differences in growth characteristics of these malignant cells and the normal bone marrow and gastrointestinal stem cells. Such an agent may also prove desirable for use in conjunction with antifolates in the chemotherapy of bacterial or protozoal infections. For example, studies indicate that 5-formylFH4 can be administered concomitantly with pyrimethamine (Daraprim) to prevent the toxic but not the therapeutic effects of this antifolate in the treatment of toxoplasmosis.

The present study was designed to assess, by radiotracer technics, whether orally administered 5-formylFH4 can be absorbed in a form that contributes to expand the body pool of reduced folate coenzymes, and so potentially to reverse the effects of antifolates.

Materials and Methods

Subjects

Four patients, each candidates for cancer chemotherapy, were selected for the studies. All had normal values for serum creatinine, blood urea nitrogen, serum vitamin B12 and routine liver-function tests. Of the four, only A.S. suffered any symptoms or clinical signs of gastrointestinal disease.

This subject, a 20-year-old man weighing 87 kg, had undergone pelvic radiotherapy for a testicular tumor three months before the study and at the time of the study complained of nausea, and had a hematocrit of 29 per cent and a serum folate level (Lactobacillus casei method) of 3.5 ng per milliliter. The remaining patients suffered from local tumors of the head and neck region, and each had a normal hematocrit. J.S., a 57-year-old man weighing 75 kg, had a serum folate of 3.9 ng per milliliter and had received 5 mg of 5-formylFH4 intramuscularly two days before the study to ensure that he was folate replete. M.S., a 74-year-old woman weighing 53 kg, had a serum folate level of 7.5 ng per milliliter. E.R., a 75-year-old woman weighing 36 kg, had a serum folate of 4.0 ng per milliliter after 3 mg of 5-formylFH4 intramuscularly two days before the study.

Materials and Procedures

Dr. Harriet Kittle, of Lederle Laboratories, kindly supplied unlabeled 5-formylFH4 (calcium leucovorin). Starting with folic acid-3H (Amersham–Searle) and formaldehyde-14C (New England Nuclear), respectively, 5-formylFH4-3H with a specific radioactivity of 250 μCi per micromole and 95 per cent radiochemical purity, and 5-formyl-14C-FH4 with specific radioactivity of 50 μCi per micromole and 97 per cent radiochemical purity were synthesized.

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and purified in our laboratory.\textsuperscript{12} Amounts of each were mixed with unlabeled 5-formylFH\textsubscript{4} for administration to fasting subjects. Hereafter, this mixture will be referred to as 5-formyl-\textsuperscript{14}C-tetrahydrofolate-\textsuperscript{3}H.

A.S. and J.S. each received a total oral dose of 10 \( \mu \)g per kilogram of body weight; M.S. received 50 \( \mu \)g per kilogram orally, and E.R. 5 \( \mu \)g per kilogram by rapid intravenous injection. Samples of serum, all urine and, in M.S., all stools, were collected through the study period. Urine and serum samples to be chromatographed contained 2-mercaptoethanol to prevent oxidation of reduced folates.

Measurement of radioactivity of samples was optimized for double-label counting of \textsuperscript{3}H and \textsuperscript{14}C, and the radioactivity due to each label, in disintegration per minute (dpm), was computed. The results were expressed as a percentage of the administered dose of each label.\textsuperscript{12}

Samples of urine were desalted by chromatography through Sephadex G-15. The resultant fractions containing radiolabeled materials, together with marker compounds, were analyzed by ion-exchange chromatography by use of DEAE-Sephadex.\textsuperscript{14} Samples of serum were similarly analyzed by ion-exchange chromatography.

\section*{RESULTS}

\section*{Oral Absorption of Radiolabels}

In all three subjects, after orally administered 5-formyl-\textsuperscript{14}C-FH\textsubscript{4}-\textsuperscript{2}H, both radiolabels appeared in the serum, peaking at about 60 minutes after administration. The time course of appearance and disappearance of labels from the serum, expressed as the percentage of the administered dose per liter of serum, is shown in Figure 1 for a representative subject (J.S.). It was similar in the other subjects, and maxima reached were 3.5 to 5.0 per cent of the dose per liter of serum, for \textsuperscript{3}H, and 2.3 to 2.5 per cent for \textsuperscript{14}C.

Figure 1 also shows the cumulative urinary clearance of radiolabels from J.S., expressed as a percentage of the administered dose. In the period one to 10 hours, the rates of urinary excretion of radiolabels were quantitatively close to, but less than, the rates of fall in concentration of serum radiolabels per liter of serum. Since the circulating plasma volume is more than double this value, and since the radiolabels probably equilibrate, in this time, through a body-fluid volume many times larger still, the urinary clearance in this period represents only a small portion of the radiolabels that have passed into and disappeared from the circulating fluid volume. Data presented below exclude the possibility of appreciable loss by gastrointestinal excretion. The largest proportion of absorbed radiolabels must therefore have been taken up into tissues.

At 24 hours after administration of labeled 5-formylFH\textsubscript{4}, J.S. received a therapeutic methotrexate infusion. In the next 24-hour period, the rate of urinary excretion of \textsuperscript{3}H increased whereas that of \textsuperscript{14}C did not, suggesting that, by this time, the two radiolabels were chemically dissociated. Moreover, the rate of urinary excretion was more than 10 times higher than that of serum disappearance for \textsuperscript{3}H, in the period of 24 to 48 hours; therefore, the increased urinary excretion of \textsuperscript{3}H that resulted from methotrexate administration was not derived from serum tritium, but at least partly from tissue tritium pools also.

Figure 2 shows that the serum appearance and disappearance data for M.S., after the fivefold higher oral dose of labeled 5-formylFH\textsubscript{4}, was similar to that for J.S., whereas the rates of urinary clearance were approximately doubled. In this patient, radiolabels were measured in the stools as long as five days after the administration of labeled 5-formylFH\textsubscript{4}. After five days the total cumulative collection of radiolabels in the stools was only 8 per cent for tritium and 5 per cent for \textsuperscript{14}C, suggesting that the radiolabels had almost completely absorbed.

For comparison, 5-formyl-\textsuperscript{14}C-FH\textsubscript{4}-\textsuperscript{2}H was administered to E.R. by rapid intravenous injection of only 5 \( \mu \)g per kilogram of body weight. At the same periods after the first hour, the concentrations of radiolabels remaining in the serum of E.R. (Fig. 3) were comparable to those of J.S. and A.S., and were half those of M.S., as might be expected only if more than 50 per cent of orally administered radiolabels were absorbed. The initial urinary clearance of intravenously administered radiolabels (Fig. 3) was higher than that after oral administration, owing to the higher serum concentrations in the first hour after intravenous administration.
graphed to identify and quantitate the folate forms with which the two radiolabels were then associated. Figure 4 represents the elution pattern of radiolabels and markers. At this time point, only about 40 per cent of the radiolabels cochromatographed with the 5-formylFH₄ marker. Almost 60 per cent of the H was now identified as 5-methylFH₄, the normal circulating serum folate, and almost 40 per cent of the C label was labilized to nonabsorbing materials, perhaps amino acids. Only 6 per cent of the H cochromatographed with p-aminobenzoylerglutamate (pABG), which would be expected to retain the largest proportion of the H were the reduced folates, labeled in the 9, 3′ and 5′ positions, degraded. Of the radiolabels chromatographed as 5-formylFH₄, the ratio of the H to C was identical with that of the administered compound.

Identification of Serum Radioactivity

Serum collected 90 minutes after intravenous administration of 5-formyl-¹⁴C-FH₄,₂H was chromato-

Figure 4. Elution Profile of Radiolabels and Marker Compounds when Serum, Collected 90 Minutes after Intravenous Administration of 5-Formyl-¹⁴C-FH₄,₂H, Was Applied to a 0.9 × 30 Column of DEAE-Sephadex and Eluted by a Concentration Gradient of Potassium Phosphate Buffer, pH 6.0.

The positions of marker compounds were monitored by ultraviolet absorbance spectrums between 240 μm and 360 μm at pH 6 and at 345 μm after incubation for one hour at 37°C at pH 1. Under the latter conditions only the positions of markers 5-formylFH₄ and 5, 10-methenylFH₄ were shown, and the absorbances due to overlapping markers could be corrected. The elution position of 10 formylFH₄ is identical with that of 5, 10-methenylFH₄.

After chromatography of serum collected 75 minutes after oral administration of 5-formyl-¹⁴C-FH₄,₂H to J.S., a different radiolabel elution pattern (Fig. 5) was obtained. No substantial amounts of either radiolabel cochromatographed with the 5-formylFH₄ marker. Almost 90 per cent of the H, and 20 per cent of the C chromatographed as 5-methylFH₄, 8 to 9 per cent of each as 10-formylFH₄ or 5, 10-methenylFH₄ (which elute together), and 70 per cent of the C was not absorbed to the column. Almost identical elution profiles were obtained when the serum specimens of A.S. and M.S. were chromatographed at similar time points.
Identification of Urinary Radioactivity

When the urine cleared by J.S. in the first zero to one hour after oral administration of 5-formyl-\textsuperscript{14}C-FH\textsubscript{5}-H was chromatographed, the total \textsuperscript{3}H was distributed principally among four compounds (Fig. 6). Less than 20 per cent of \textsuperscript{3}H was eluted in the position of 5-methylFH\textsubscript{5}, whereas 40 per cent was identified as 5-formylFH\textsubscript{5}, and about 40 per cent eluted as a complex peak including both pABG and 10-formylFH\textsubscript{5} or 5, 10-methenylFH\textsubscript{5}. The small amount of \textsuperscript{14}C absorbed to the column was distributed between the latter folates and 5-formylFH\textsubscript{5}. Urines collected from J.S. at later periods, two to four hours (Fig. 7) and four to eight hours, progressively contained much less \textsuperscript{3}H identifiable as 5-formylFH\textsubscript{5} and 5-methylFH\textsubscript{5}, whereas the excretion of \textsuperscript{3}H identifiable as 10-formylFH\textsubscript{5} or 5, 10-methenylFH\textsubscript{5} was maintained. Urines collected in corresponding periods from A.S. and M.S. contained radiolabels similarly distributed. In all the urine samples, the proportion of total tritiated urinary folates identifiable as 5-methylFH\textsubscript{5} varied directly with the serum concentration of 5-methylFH\textsubscript{5}-H, between the limits observed, which were 6 per cent at 7.5 and 70 per cent at 120 ng per milliliter.

At 99 hours and again at 101 hours after the oral administration of 5-formyl-\textsuperscript{14}C-FH\textsubscript{5}-H, M.S. received 6 mg of unlabelled 5-formylFH\textsubscript{5} intramuscularly. The urine cleared in the next 20 hours contained no radiolabeled 5-formylFH\textsubscript{5}. Rather, 30 per cent of \textsuperscript{3}H chromatographed as 5-methylFH\textsubscript{5}, 70 per cent of \textsuperscript{3}H, and 60 per cent of \textsuperscript{14}C as 10-formylFH\textsubscript{5} or 5, 10-methenylFH\textsubscript{5}, and 40 per cent of \textsuperscript{14}C was not absorbed and presumably converted to nonfolate compounds.

After intravenous administration of 5-formyl-\textsuperscript{14}C-FH\textsubscript{5}-H to E.R., urine collected in the period of zero to one hour contained \textsuperscript{3}H and \textsuperscript{14}C, both of which were distributed almost entirely as 5-formylFH\textsubscript{5} and were present in the same ratio as in the administered material. Urine collected in the period of two to four hours from E.R. differed only in that 30 per cent of the radiolabels chromatographed as 10-formylFH\textsubscript{5} or 5, 10 methenylFH\textsubscript{5}.

**DISCUSSION**

These data clearly show that orally administered 5-formylFH\textsubscript{5} was well absorbed so as to expand the
The results of chromatography of urine samples after administration of 5-formyl-1-14C-FH4 suggest that there is a fairly constant urinary clearance of folates in the forms of 10-formylFH4 or 5, 10-methylFH4. The fraction of total radiolabeled urinary folates found as 5-methylFH4 appeared proportional to the serum concentration of radiolabeled 5-methylFH4. In contradistinction, large amounts of radiolabeled 5-formylFH4 were found in the urine in the early period (zero to two hours) even when, as after oral administration of 5-formylFH4, no label was clearly measurable in the serum. In similar studies in our center in which radiolabeled 5-methylFH4 was administered, we never observed labeled 5-formylFH4 in the urine. It appears, therefore, that the kidney may have both regulatory and metabolic roles in the excretion of folates: serum 5-methylFH4 may be conserved by the kidney in preference to 5-formylFH4 and the most constant urinary folates, 10-formylFH4 and 5, 10-methylFH4, differ from the serum folate.

These data considerably extend preliminary reports that orally administered 5-formylFH4 is able to raise the level of serum folates assayed by L. casei. Our studies justify the use of oral administration of 5-formylFH4 to reverse the toxic and therapeutic effects of methotrexate and other antifolates. Preliminary studies in mice as well as man indicate that oral leucovorin may be useful in regimens for the chemotherapy of cancer and infectious diseases.

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REFERENCES