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Human Pharmacokinetics of 5-Formyl Tetrahydrofolate

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HUMAN PHARMACOKINETICS OF

5-FORMYL TETRAHYDROFOLATE

by

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

1981

ABSTRACT

The purpose of this study was to determine the
pharmacokinetics of calcium leucovorin in normal subjects
and in patients undergoing High-Dose methotrexate therapy
for the treatment of neoplastic disease. By characterizing
the disposition of leucovorin it was hoped that the the
efficacy of the methotrexate therapy might be improved,
while at the same time, the incidence of toxicity could be
diminished.

An assay for the separation and quantification of
leucovorin in serum was developed. The reduced folate was
extracted from serum using reverse-phase chromatographic
minicolumns and a paired-ion reagent. Leucovorin was
eluted from this system in methanol and evaporated to
dryness under nitrogen. The extracted samples were stable
for 3 days when stored at -4°C. High pressure liquid
chromatography was used to separate leucovorin from the
extracted serum components using a reverse-phase,
paired-ion mode. Ammonium phosphate, dibasic, was found to
be a suitable pairing agent. The recently developed
radially compressed reverse-phase columns were found to
provide superior resolution of serum components than was
possible with conventional stainless steel columns. The

effects of flow rate, pH, column temperature, and buffer concentration on the separation of leucovorin were investigated.

Electrochemical detection was used to quantify the reduced folate in the extracted serum samples. An applied potential of 0.8 volts was used to produce a sensitive and selective means of detecting leucovorin. The apparent lower limit of sensitivity under the conditions employed was 12 ng leucovorin. The effects of pH, buffer concentration, methanol concentration, and mobile phase flow rate on detector response were investigated.

The protein binding of leucovorin and its major metabolite 5-methyltetrahydrofolate to human serum albumin was determined. Over the concentration range of $5.0x10^{-7}M$ to $1x10^{-4}M$ neither folate saturated the available binding sites. Leucovorin binding was a constant 70%, while methyltetrahydrofolate decreased slightly over this range. When both folates were present in the albumin solution, there was a significant decrease in the degree of binding of each. Methotrexate did not affect the binding of leucovorin or its metabolite.

The pharmacokinetics of leucovorin was determined in 6 normal subjects and in 5 cancer patients. There were no significant differences in the kinetic parameters calculated from either group. The disposition of leucovorin is best characterized by a two compartment open model. An initial half-life of 8.79 ± 4.44 minutes and a

second, slower elimination phase of 231.46 ± 31.76 minutes was determined. The volume of the central compartment was calculated as 5.49 ± 3.53 Liters. Leucovorin serum concentrations in 3 cancer patients receiving High- Dose methotrexate were determined to be 3 to 11 times greater than was predicted using methotrexate pharmacokinetic parameters.

This study clearly demonstrates that there are significant differences in the pharmacokinetics of leucovorin and methotrexate. The data presented in this study suggests that it might be possible to reduce the amount of leucovorin administered to patients being treated with methotrexate. This may be expected to improve the efficacy of the antimetabolite therapy without additional risk of toxicity.

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I. INTRODUCTION

The folate antagonist methotrexate (MTX) is an effective antineoplastic agent widely used for the treatment of choriocarcinoma, osteosarcoma, and a variety of head and neck tumors (Stoller et al., 1977). It has also been used in non-neoplastic diseases such as psoriasis (VanScott et al., 1964) systemic lupus erythematosus (Swanson and Schwartz, 1976), and psoriatic arthritis (Fosdick, 1968). Methotrexate acts at the cellular level by reversibly binding the enzyme dihydrofolate reductase, preventing the reduction of dihydrofolic acid to tetrahydrofolic acid, thereby depleting the reduced folate pool necessary for the transfer of 1-carbon units in a number of biochemical pathways. As a result, DNA synthesis, purine synthesis, and several amino acid interconversions are severely inhibited (Bleyer, 1977). Methotrexate's toxicity to normal and malignant cells is dependent on both the administered dose and the duration of exposure (Djerassi, 1975).

Since Farber first reported the successful use of an antifolate for the treatment of leukemia (1948), MTX dosage

schedules have changed dramatically. First used daily and given orally, progressively larger and more frequent doses (orally, intramuscularly, and intravenously) have provided consistently improved clinical response.   Djerassi has reported success in treating previously resistant tumors with MTX dosages in the range of 100-200 mg per m² where as little as 3 mg per m² had been employed. Bleyer (1977), Goldin (1978), and Djerassi (1975), have all discussed the historical background of high dose MTX therapy.

The goal of MTX research has been to identify the maximum dose and frequency of administration tolerated by normal tissues that will still be effective against tumor cells.   Toward this end the pharmacokinetics of MTX have been extensively studied (Bischoff et al., 1971, Reich et al., 1977 and Huffman et al., 1973) and the absorption, distribution, metabolism, and excretion of the drug in both animals and humans are well understood.

Leucovorin (citrovorum factor, 5-formyl THF, LEU) is a reduced folate, capable of supplying 1-carbon units in the biosynthetic pathways inhibited by MTX, since its further reduction does not depend on dihydrofolate reductase. Although the mechanism by which leucovorin "rescues" a host organism is unknown, the prevailing theory postulates a competition for a common transport mechanism regulating entry into cells (Chabner et al., 1975). As early as 1949 Burchenal et al. (1949,1950) had reported the prevention of therapeutic effect of MTX on mouse leukemia with the

administration of LEU or pteroylglutamic acid. Goldin et
al., (1953,1954,1955,1966) and Bernbaum et al., (1965)
working with leukemic mice produced results suggesting that
proper administration of LEU subsequent to MTX could
prevent toxicity in normal cells without protecting the
malignant cells. Shoenbach et al., (1950) reported a case
in which LEU was successfully used to reverse MTX-induced
toxicity in a patient. However, it was not until the work
of Djerassi et al., (1966,1967,1968,1970) and Lefkowitz et
al., (1967) that controlled studies clearly demonstrated
the role of LEU in preventing MTX-induced toxicity. Since
the publication of these works, the cancer literature has
been flooded with reports of improved clinical response in
patients treated with high dose MTX-LEU rescue regimens.
However, there is still considerable debate regarding the
optimal dose and schedule of LEU which should be employed.
Penta (1975), in a review of IND protocols discussed ten
different treatment schedules while Nixon (1979) described
a 100-fold range in the doses of LEU employed and labeled
current regimens as arbitrary.

Leucovorin has been assayed in human plasma
biologically (Mehta et al., 1978), radiochemically
(Rothenberg et al., 1979), radioimmunologically (Raso,
1977), spectrophotometrically (Chapman, 1978), and by
administration of radiolabelled drug (Nixon and Bertino,
1972). The applicability to pharmacokinetic analysis of
each of these methods, however, has severe limitations.

Spectrophotometric methods are not sufficiently sensitive to reduced folates; microbiologic assays are generally not precise enough for pharmacokinetic analysis; radiochemical and radioimmunologic techniques suffer from a lack of reproducibility; and the use of radiolabeled drug is inappropriate for the general patient population.

The pharmacokinetic data derived from these methods is of limited value for two reasons. First, these studies have been carried out at relatively low doses and in the absence of methotrexate, and, therefore, they do not reflect the clinical conditions under which LEU is employed (1979). Second, although plasma concentrations of LEU have been determined, these studies were not adequately designed with regard to sampling time and duration of sampling for complete pharmacokinetic analysis.

The purpose of this work is twofold: (1) to develop a rapid, sensitive, and reproducible assay for LEU in plasma and; (2) to characterize the disposition of LEU in normal subjects and in patients receiving high dose MTX therapy. Determination of the pharmacokinetic parameters governing LEU distribution and elimination should make possible the optimization of LEU rescue protocols without adversely affecting the anti-neoplastic activity of MTX.

II. LITERATURE SURVEY

A. Folate Chemistry

The folates are a series of compounds containing the pteroic acid nucleus, conjugated with one or more L-glutamic acid molecules (Figure 1) (Blakely, 1969). Folic acid, N-[p-[[(2-Amino-4-hydroxy-6-pteridinyl)- methyl]amino] benzoyl]glutamic acid or pteroylglutamic acid (PGA), is a partially oxidized, stable form of folate found in pharmaceutical preparations but rarely in nature (Nixon, 1979). Folic acid is rapidly reduced, first to dihydrofolate (DHF), then to tetrahydrofolate (THF) by the enzymes dihydrofolate reductase and tetrahydrofolate reductase, respectively (1970). Tetrahydrofolate serves as an intermediate carrier of formyl, methyl, and hydroxymethyl one- carbon units, which are usually attached in the N^5,N^{10}, or N^{5-10} positions. These reduced folate forms are found in nearly all natural foods including mammalian cells (Blakely, 1969). The so-called "folate coenzymes" participate in a large number of enzymatic reactions in which one-carbon units are transferred from one metabolite to another or alternatively may be interconverted to other coenzyme forms (1970). Figure 2 represents the metabolic pathways and interconversions followed by folic acid and various folate coenzymes which result in the conversion of glycine to serine, homocysteine to methionine and deoxyuridine monophosphate (dUMP) to thymidylate monophosphate (dTMP) and ultimately the
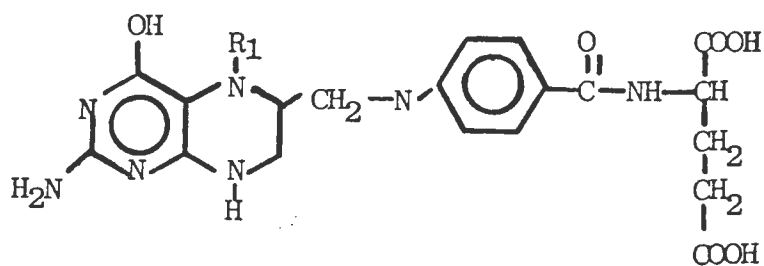
$R_1 = CH_3$: 5-methyltetradrofolic acid, METHF

$R_1 = CHO$; Leucovorin, 5-Formyl THF
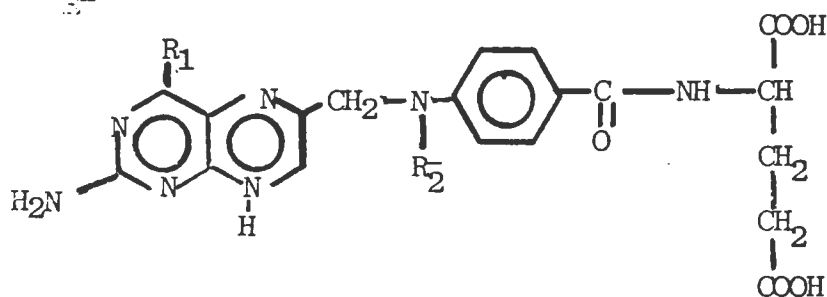
$R_1 = OH$, $R_2 = H$: Folic Acid, FA

$R_1 = NH_2$, $R_2 = CH_3$: Methotrexate, MTX

Figure 1:   Folate Structures
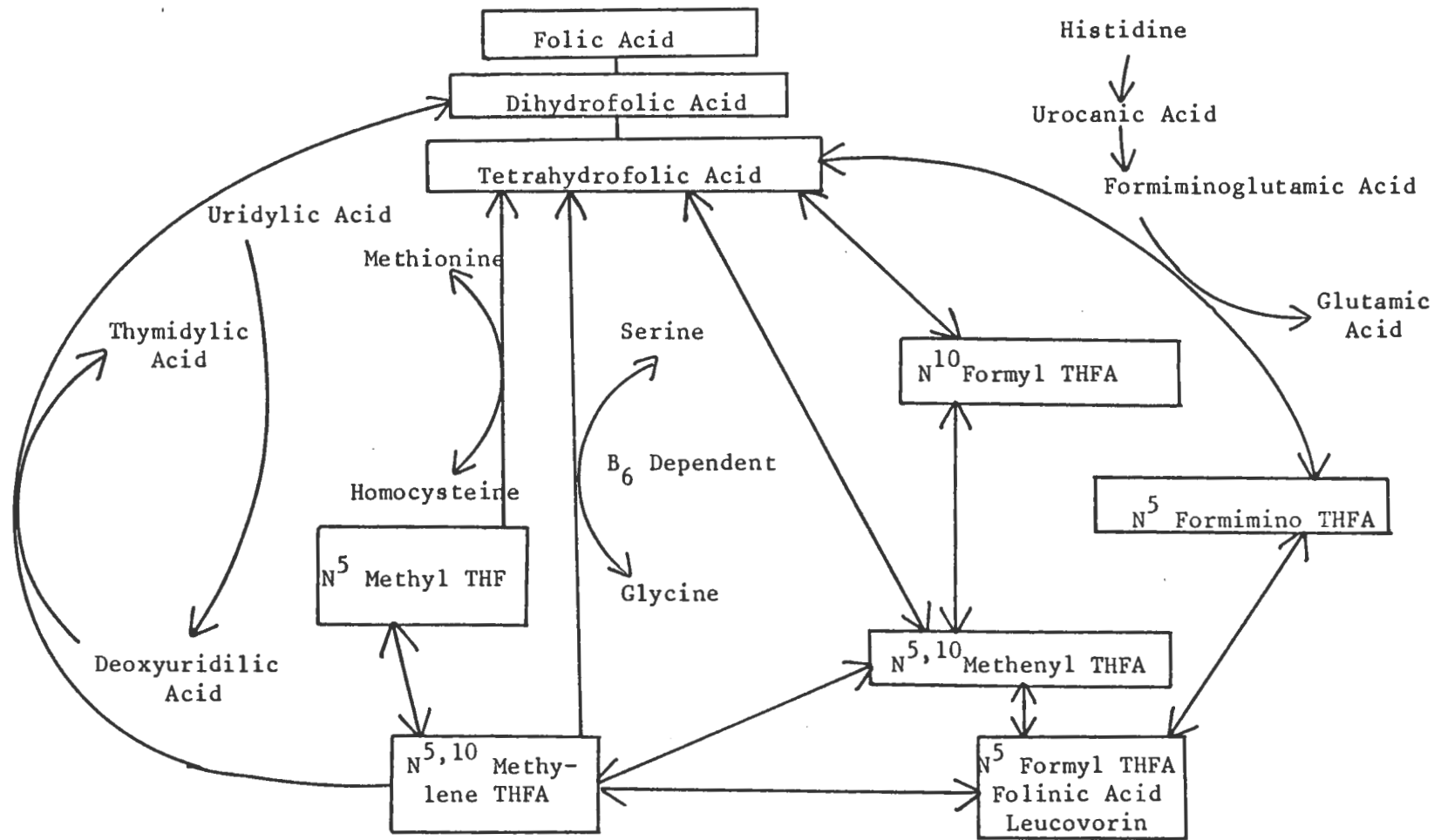
Figure 2: Metabolic Pathways Involving
Reduced Folate Coenzymes

The diagram contains the following labeled boxes and terms:

Folic Acid

Dihydrofolic Acid

Tetrahydrofolic Acid

Histidine

Urocanic Acid

Formiminoglutamic Acid

Glutamic Acid

Uridylic Acid

Methionine

Serine

N^{10} Formyl THFA

Thymidylic Acid

B_6 Dependent

N^5 Formimino THFA

Homocysteine

N^5 Methyl THF

Glycine

Deoxyuridilic Acid

$N^{5,10}$ Methenyl THFA

$N^{5,10}$ Methylene THFA

N^5 Formyl THFA
Folinic Acid
Leucovorin

synthesis of DNA. An excellent review of the biochemistry
of folic acid, folate coenzymes, and related pteridines has
been published (Blakely, 1969).

B.   Leucovorin

1.   Physical-Chemical Properties

Folinic acid,   N-[p-[[(2-amino-5-formyl-5,6,7,8,-
tetrahydro -4- hydroxy -6-
pteridinyl)methyl]amino]benzoyl]-L glutamic acid, molecular
weight 511.513, is the formyl derivative of THF. It is one
of the forms of reduced folate coenzymes found in the body.
(Blakely, 1969).   The term leucovorin generally refers to
the chemically synthesized material containing both the -L
and l diastereomers, while "folinic acid" or "citrovorum
factor" applies to the biologically active l isomer only
(Ponte et al., 1979).

Folinic acid (also called 5-formyl tetrahydrofolate)
is a yellowish-white or yellow, odorless, microcrystalline
powder (Blacow, 1972).   It is sparingly soluble in water
and practically insoluble in alcohol.   The pH of a
saturated aqueous solution is 2.8 to 3.0, at which partial
decomposition takes place.   Three pKa values have been
reported for the free acid; 3.1, 4.8, and 10.4. The first
two are attributed to the glutamyl carboxyl groups and the
third to the C-4 hydroxyl group (Ponte et al., 1979). The
greatest stability is at neutral or mildly alkaline pH
(Merck Index, 1976). The commercially available salt form

of folinic acid, calcium leucovorin pentahydrate, molecular weight 601.513, is freely soluble in water but remains practically insoluble in alcohol (Merck Index, 1976). All the reduced folates are heat labile; folinic acid is, however, the most stable, and for this reason it is the only commercially available pharmaceutical preparation of a reduced folate coenzyme (Kastrup and Boyd, 1980). An excellent review of the physical properties of LEU including infrared spectrum, proton magnetic resonance spectrum, C13 magnetic resonance spectrum, mass spectrum, x-ray crystallography, optical rotation and circular dichroism has recently been published (Ponte et al., 1979).

2. LEU Isolation and Identification

During a survey of organisms suitable for a microbiological assay of alanine, Sauberlich and Baumann(1948) discovered that _Leuconostoc citrovorum_ 8081 failed to grow on a medium that had proved suitable for similar assay organisms. They also reported that various "antianemic" crude liver and yeast extracts promoted the growth of the organism and did so at a substantially faster rate than did folic acid.

Keresztesy and Silverman (1950) reported the partial purification and concentration from liver of a "citrovorum-factor" so named because of its ability to stimulate the growth of _L. citrovorum_. Brockman et al. (1950) and Bardos et al., (1949) reported the preparation

of   a compound resulting from the formylation and reduction
of   pteroylglutamic   acid   (folic acid), whose activity was
similar to the extracted material.

Keresztesy   and   Silverman   (1951),   and Silverman and
Keresztesy   (1951),   compared the activity of the synthetic
compound   with   the liver extract and found that the latter
was   approximately   twice   as   active,   relative   to   L.
citrovorum   growth.   It   is   now   known that the synthetic
material   contains   both   the   inactive   dL   and   active lL
diasteriomers.   The   naturally occuring extracted material
contains   only   the   active lL form, it is clear that, on a
weight-weight   basis,   it   would   be   more   active than the
synthetic material.

Pohland   et   al.,   (1951),   proposed   a   structure for
folinic   acid-sf   ("sf"   designates   a   synthetic folate, as
opposed   to   a   liver   extract   as,   5-formyl-5,6,7,8-
tetrahydropteroyl glutamic acid, (Figure 1).   The synthesis
from   either pteroylglutamic acid or formylpteroyl glutamic
acid,   (Roth, 1952) as the chemistry (Cosulich et al., 1952)
of LEU have since been reported in detail.

3.   Biochemical Role of LEU

Leucovorin was the first naturally occuring derivative
of   THF   to   be   isolated,   identified,   and   synthesized.
(Blakely,   1969).   Although   the   mechanism   of   its
biosynthesis remains uncertain, three have been
proposed   (Figure   3):   (1) the reverse of an ATP dependent

conversion of LEU to N^5, N^{10}, methenyl THF and ADP + Pi; (2) the reverse of a similar ATP dependent conversion of leucovorin to N^{10} formyl THF + ADP + Pi; and (3) the formation of LEU and L-glutamate from THF and formylglutamate. (Donaldson and Keresztesy, 1959) Although the first two mechanisms are possible, they are not favored thermodynamically, and the third is limited by the quantity of formylglutamate available (Blakely, 1969).

Leucovorin is not believed to be involved in the direct transfer of one-carbon units in the biosynthesis of amino acids or the purine and pyrimidine units of DNA. (Blakely, 1969) Rather, it exists in equilibrium with the N^5, N^{10} methylene, methenyl and N^{10} formyl folate coenzymes which are directly involved in several syntheses (lehninger, 1970).

The major role for LEU, although indirect, is in the synthesis of serine from glycine (Kislivk and Sakami,1955; Alexander and Greenberg, 1955; Blakely, 1954; and Blakely, 1957). Leucovorin confers the one-carbon (formyl) group on N^5, N^{10}-methylene THF converting it to THF by donating the formyl group to glycine, resulting in the formation of serine (Figure 2).

The formyl group originating from LEU also participates in two reactions important to purine biosynthesis. N^5, N^{10} methenyl THF donates the formyl 1-carbon unit in the conversion of glycinamide ribonucleotide (GAR) to formylglycinamide ribonuclieotide

1. 5-Formyl THF + H$^+$ $\rightleftharpoons$

        5,10 Methenyl THF +H$_2$O

2. 5-Formyl THF + ATP $\rightleftharpoons$

        10-Formyl THF + ADP + Pi

3. 5-Formyl THF + L-glutamate $\rightleftharpoons$

        THF + N-formyl-glutamate
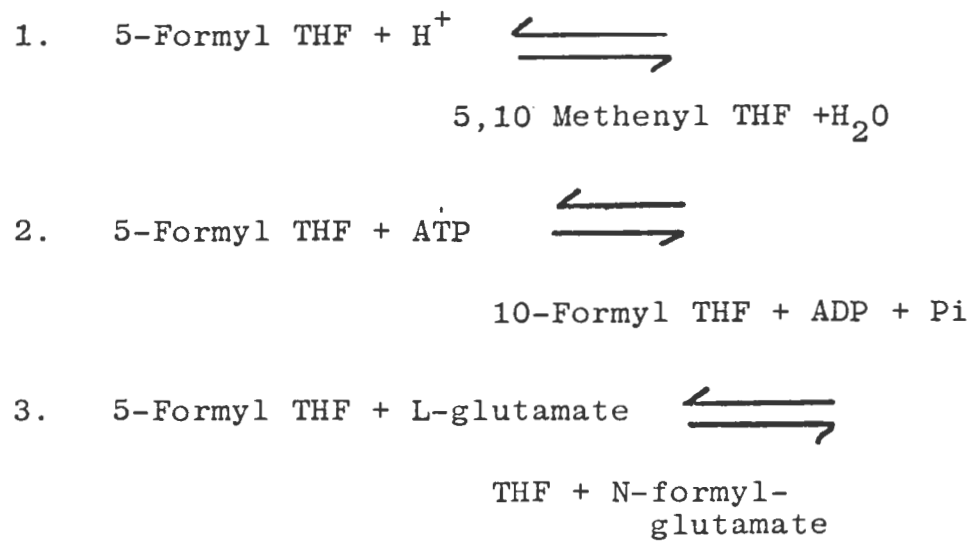
Figure 3: Leucovorin Biosynthetic Pathways

(fGAR) (Calabresi and Parks, 1975). N^{10} formyl THF donates its formyl group in the conversion of 5-aminoimidazole carboxamide ribonucleotide (AICAR) to 5-formamido-imidazole-4-caboxamide ribonucleotide (FAICAR) (Lehninger, 1970). The synthesis of both fGAR and FAICAR are important steps in the biosynthesis of inosinic acid, and ultimately purine mononucleotides (Lehninger, 1970).

4. Clinical Applications of LEU
a. Megaloblastic Anemia

Leucovorin may be used in the treatment of folate deficient megaloblastic anemias resulting from pregnancy, sprue, or nutritional deficiencies (A.S.H.P., Leucovorin Monograph, 1980). However, LEU offers no advantage over folic acid in treating these disorders and is considerably more expensive. LEU may be useful in treating conditions of folate deficiency anemias in which the ability to reduce folic acid to the various coenzyme forms is impaired (A.S.H.P., Leucovorin Monograph, 1980). Anemia associated with acute portal cirrhosis (alcoholic or Laennecc's cirrhosis) often does not respond to folic acid therapy (Patek, 1969). This phenomena has been attributed to an inability to produce reduced coenzyme forms rather than an actual lack of folic acid itself. It has been suggested in such cases that because of its ready conversion to other reduced coenzyme forms LEU be used to treat the anemia (Patek, 1969).

Tauro et al., (1976) reported two cases of megaloblastic anemia in infants resulting from a congenital deficiency of dihydrofolate reductase which prevented the reduction of dihydrofolate to THF. Both infants showed "satisfactory clinical response" following parenteral LEU therapy.

b. Reversal of Antifol Toxicity

The major clinical application of leucovorin is as an antidote for the folate antagonists pyrimethamine, trimethoprim, and methotrexate (A.S.H.P. Leucovorin Monograph, 1980). Pyrimethamine is a folic acid antagonist used in the treatment of chloroquin-resistant malaria caused by susceptible strains of plasmodia (Waxman and Herbert, 1969) and may be used to treat ocular toxoplasmosis (Giles et al., 1964). It can produce megaloblastic anemia which, reportedly, has been reversed by LEU but not by folic acid (Giles et al., 1964; and Waxman and Herbert, 1969).

Trimethoprim acts as an inhibitor of dihydrofolate reductase, blocking the production of THF (Kastrup and Boyd, 1980). Toxicity resulting from trimethoprim therapy could presumably be treated by administering LEU and by-passing the point of enzyme blockade.

5. Methotrexate

The use of LEU in conjunction with methotrexate (MTX) therapy represents the most important and widely studied application of a reduced folate in humans.

a. Physical-Chemical Properties

Methotrexate, (amethopterin, MTX), N-[p-[[(2,4-Diamino-6-pteridinyl)methyl]-methyl amino]benzoyl]glutamic Acid, molecular weight 454.45, is a yellow powder, structurally related to folic acid, Scheme 1 (Merck Index, 1976). MTX is practically insoluble in water, alcohols, chloroform, and ether. It is freely soluble in dilute solutions of alkali hydroxides and carbonates (Blacow, 1972). The solubility of MTX has been reported to be 2.2×10^{-3} M in urine at pH 5.1 and 2.2×10^{-2} M at pH 6.9 (Bleyer, 1977). MTX has two pKa's; 4.8 and 5.5, attributable to the two carboxyl groups of the glutamic acid portion of the molecule (Bleyer, 1977).

b. Biochemistry and Pharmacology

Methotrexate, is one of several derivates of 2-4 diaminopteridine which inhibit dihydrofolate reductase (Plenderheith and Bertino, 1975), the enzyme responsible for the reduction of dihydrofolate to tetrahydrofolate. This enzyme block results in the depletion of THF and consequently the supply of folate coenzymes available for

thymidylate and purine synthesis and the formation of
serine and methionine (Calebresi and Parks, 1975).
Thymidylate synthesis is reportedly the most sensitive to
depletion of THF stores (Plenderheith and Bertino, 1975).
As a result, DNA synthesis is affected to a greater degree
than is RNA synthesis. Because DNA synthesis is most
severly affected, MTX's mechanism of action is considered
to be cell cycle specific, acting in the "S-phase" of
mitotic activity (Bleyer, 1977). In this manner, those
tissues undergoing rapid cellular turnover with a high
percentage of cells in "S-phase" are most susceptible to
the effects of MTX (Bleyer, 1977). The binding of MTX to
the enzyme is considered to be of a "tight" but reversible
nature (e.g., it is a competetive stoichiometric inhibitor
(Plenderheith and Bertino, 1975; Blakely, 1969). Johns et
al., (1964), demonstrated that H^3-MTX bound to
intracellular enzyme in man could be displaced to
extracellular spaces by unlabelled MTX or by dihydrofolate.

In addition to MTX's affect on dihydrofolate
reductase, Borsa and Whitmore (1969), have suggested that
MTX may also act by direct inhibition of thymidylate
synthetase.

Because the mechanisms for methionine and serine
synthesis are disrupted by MTX, protein synthesis may also
be affected (Bleyer, 1977). The author suggests that by
this mechanism MTX arrests cells in the G1 phase of
development and in so doing, protects those which survive

MTX exposure by slowing their progression in the S-phase. If however, protein synthesis is disrupted for long periods, cells will, of course, be seriously injured.

Goldman (1974) reported that for maximal suppresion of DNA synthesis, free intracellular methotrexate concentrations must be greater than those required to stoichiometrically bind with dihydrofolate reductase. The additional MTX is considered necessary to bind any new reductase which may be synthesized, and to saturate a (proposed) second class of enzyme binding sites with a much lower affinity for MTX (Goldin, 1974,1975). Chabner et al. (1973) and Strauss and Goldstein (1943), however, suggest that these findings are compatible with interactions between MTX and a single, high affinity site. In either case the requirement for free intracellular MTX has been established. It is now generally recognized that the antineoplastic activity of MTX is dependent upon maintaining free intracellular levels above a threshold value which is specific for various tissues or tumor types) for prolonged periods and not simply peak plasma MTX concentrations (Chabner and Young, 1973).

        c. High-Dose Methotrexate-Leucovorin
            Rescue Regimens

Farber (1948) reported the first successful treatment of cancer with a folic acid derivative when aminopterin was used to produce temporary remission of acute leukemia in

children. Similar results were reported by Sacks et al.,
(1950), with both aminopterin and MTX, in low daily doses
of approximately 0.1 mg kg^{-1}. However, Schoenbach et al.,
(1950) described a folic acid deficiency, caused by the
antimetabolites, which was manifested by "gastrointestinal
cramps, diarrhea, hemorrhage, ulceration of the palate and
oral mucous membranes and leukopenia which may progess to
an aplastic anemia and alopecia". Because the appearance
of these toxic manifestations was frequently delayed, the
cumulative toxic dose might have been exceeded before
symptoms occured. As a result, it has been suggested
(Shoenbach et al., 1950) that sub-therapeutic doses of MTX
might have been employed in an effort to prevent toxicity.

Early treatment schedules involving MTX usually
required daily oral doses of 1 to 5 mg., depending on the
age of the patient (Acute Leukemia Group B., 1965).
Working in mice with leukemia 1210, Goldin et al., (1956)
experimented with different dosage schedules and found that
drug administration every fourth day produced better
results than did either more frequent or less frequent
administration. Based on these data, the Acute Leukemia
Group B, (1965) compared intermittent parenteral
administration to daily oral administration of MTX and
found the new treatment schedule improved survival time in
childhood leukemic patients. However, the twice weekly
parenteral MTX doses of 30 mg m^{-2} produced significantly
more toxic symptoms than did daily oral dose of 3 mg m^{-2}.

Papac et al., (1967) used intravenously administered MTX, 0.8 mg kg⁻¹ in a single bolus injection every four days to treat squamous cell carcinoma. Although good clinical response was achieved there were frequent incidences of hematopoietic disorder, thereby limiting the duration of treatment.

The toxic manifestations produced by MTX could not be reversed clinically with folic acid, crude liver extracts or vitamin B12. Folic acid, when given prior to MTX (under certain conditions), has been used to prevent antifol toxicity but the antineoplastic activity of MTX was also prevented (1950). When administered simultaneously or after MTX injection, folic acid afforded no protection from the toxic manifestations of antimetabolite therapy.

The failure to reverse or eliminate MTX's toxicity was a severe deterrent to optimal anti-metabolite therapy which had been proven beneficial in treating various forms of leukemia. Nichol and Welch (1950), reported that LEU had been used successfully to prevent the toxicity of aminopterin in both mice and rats. Schoenbach et al., (1950) described the use of LEU to reverse aminopterin or amethopterin-induced mucositis in two patients under treatment with daily oral doses. It was also reported that it was possible to continue the antifol therapy while administering LEU to treat the induced toxicity.

Preclinical studies by Goldin et al., (1953), demonstrated that simultaneous administration of LEU

reduced both the toxicity and antitumor effects of MTX in both non-tumor and tumor-bearing mice. However, when it was administered in this manner, LEU actually reduced the survival time of mice that survived the initial drug toxicity. Further studies with murine L1210 leukemic mice confirmed that the prevention of toxicity is highly schedule-dependent and that delayed administration of LEU might be therapeutically advantageous (Goldin, Vendetti, et al., 1955). Goldin et al., (1953) demonstrated that delaying the administration of LEU twenty-four hours from the time of MTX dosing increased the specificity of action of amethopterin, reducing toxicity and allowing the employment of higher MTX doses. Goldin et al., (1966) also reported that through proper scheduling of MTX with LEU rescue, it was possible to administer as much as 1000 mg Kg^{-1} to tumor-bearing mice without toxicity, a dose which, without LEU, was toxic to all animals. The work of Goldin et al., (1953,1955,1956,1966) clearly demonstrated the increase in the margin of safety for MTX when used in conjunction with the delayed administration of LEU.

On the basis of these findings, the LEU-rescue concept was extended to humans. Clinical studies in the treatment of metastatic osteogenic sarcoma (Jaffee et al., 1974), head and neck tumors (Capizzi et al., 1970), leukemia (Djerassi, 1967), and other solid tumors (Djerassi et al., 1972, Levitt et al., 1973) have since demonstrated the activity of high dose MTX- LEU rescue regimens. To date,

the dosages of LEU and MTX used, the length of the MTX
infusion, and the scheme for LEU rescue vary widely between
protocols and have been established largely on an empiric
basis (Stoller, 1978).

Recent work in tissue cell culture and sarcoma 180
murine tumor models has clearly shown a relationship
between extracellular concentrations of MTX and LEU and the
effectiveness of rescue. Penedo et al., (1976)
demonstrated a competetive reversal of MTX cytotoxicity
such that the toxicity produced by $10^{-7}M$ MTX was completely
reversed by equimolar concentrations of LEU, but with
higher MTX concentrations, relatively more LEU was
required. For example, $10^{-5}M$ MTX produced cytotoxic
effects in cell culture which were not reversed until LEU
concentrations reached $10^{-3}M.$; furthermore, it was not
possible to reverse the toxicity resulting from MTX
concentrations greater than $10^{-4}M.$ (Pinedo et al., 1976).
Sirotnak et al., (1976), demonstrated a dose and
schedule-dependence for LEU rescue during in plasma high
dose MTX therapy of ascitic forms of L1210 and sarcoma 180.
The authors noted that "schedules with very delayed
'low-dose' LEU rescue following lethal doses of MTX were
highly effective in preventing toxicity and achieved a
pronounced antitumor effect in both ascites tumor models".
The best results were reportedly obtained when LEU was
withheld until 16 to 20 hours following MTX, then
administered every 2 hours for 5 doses. MTX was

administered at a dose of 400 mg Kg^{-1} and LEU at 12 mg Kg^{-1}. Further increases in MTX doses did not substantially increase the anti-tumor activity; while progressively increasing the LEU dosage reduced both the toxicity and anti-tumor effect. These data clearly suggest that manipulation of LEU dosage and schedule of administration can improve the therapeutic index of high dose MTX therapy.

Based on these observations, several clinical trials involving high dose MTX with LEU rescue have been undertaken. Table I shows some of the protocols which have been developed. Stoller et al., (1978) recently published a study (63) in which LEU was administered intravenously 30, 36, and 42 hours following an 18 hour MTX infusion. Rather than use a standard LEU dose, the authors individualized the amount of LEU administered based on the plasma MTX clearance in the patient undergoing treatment. Plasma samples were obtained from patients and, using appropriate pharmacokinetic principles, the MTX plasma level at 30 hours post infusion was calculated. In this manner it was possible to calculate an appropriate LEU dosage as well as identify as high risk patients, those individuals who were not clearing MTX adequately. Although Pinedo et al., (1976) had shown that at low MTX levels (i.e. less than 10^{-7}M) equimolar concentrations of LEU were sufficient to effect rescue, Stoller et al., (1978) elected to use a dose of LEU tenfold higher as a safety factor. The volume of distribution, distribution or elimination

TABLE I

METHOTREXATE PROTOCOLS

Methotrexate Dose (Lowest/Highest)	Delay Time (Hrs.) for CF Rescue	Dose & Schedule of CF Rescue
#1 – 100 mg/12g	1.5	6-25 mg q6h x 6-24 – IM
#2 – 240 mg/m^2/*	NONE	40 mg/m^2 over 6 hrs, then 25 mg q6h x 4 – IM
#3 – 200/500 mg/kg	4	9 mg q6h x 12 – Oral
#4 – 200 mg/kg	2	12 mg, wait 6 hrs, then 12 mg q6h x 11 – IV, Oral

*Not Specified

characteristics of LEU have not been reported, therefore, these authors assumed that, since MTX and LEU are structurally related, LEU pharmacokinetic parameters were identical to those of MTX. Using this approximation, they were able to minimize the LEU dosages employed and reduce the incidence of myelosuppresion in patients to less than 3 percent, thus demonstrating that careful pharmacokinetic monitoring could improve the therapeutic index of MTX.

d. MTX Pharmacokinetics

i. Absorption

Henderson et al. (1965) reported that H^3-MTX, administered as a solution, was completely absorbed at a dose of 0.1 mg Kg^{-1}. Freeman (1958) also found the drug to be completely absorbed following the oral administration of 30 mg. of MTX. Peak plasma levels are reportedly achieved within one to four hours of oral doses. Wan et al. (1974) described evidence which suggested biliary secretion and subsequent reabsorption of MTX. These authors found peak plasma concentrations following a 30 mg m^{-2} oral dose to be lower than those achieved when the identical dose was administered intravenously. Furthermore, the area under the plasma concentration -time curve was approximately 47% that of the intravenous dose. These authors also determined, via urinary excretion data, that approximately 88% of the administered dose was absorbed. Burchenal et al. (1956) reported a lower figure, approximately 40% of

the drug was recovered in urine within 24 hours of a 5 mg. oral dose.

When MTX was administered orally at a dose of 80 mg m^{-2}, absorption was erratic and incomplete (Henderson et al., 1965 and Wan et al., 1974). At this dose only 31% of the drug was excreted in the urine, while fecal excretion increased from approximately 1% following an intravenous dose to almost 30%.

These data have led Bleyer (1977) to conclude that the absorption of MTX may involve a saturable intestinal absorption mechanism, possibly including an active transport mechanism.

In addition to absorption from the gastrointestinal tract, MTX absorption into plasma from the central nervous system has been reported. MTX is used for the treatment of meningial leukemia and may be administered via ventricular or lumbar injection (Shapiro et al., 1975). Jacobs et al. (1975) reported that the administration of 10 to 15 mg m^{-2} intrathecally, resulted in plasma concentrations above 10^{-8} M, two to three times longer than the same dose administered intravenously.

ii. Distribution

The disappearance from plasma of intravenously administered MTX has been described both as biphasic and triphasic. Huffman et al. (1973) reported an initial half-life of 0.75 ± 0.11 hr., most likely due to

distribution. Stoller et al. (1975) and Pratt et al. (1974) failed to detect such an early distributive phase because of inadequate plasma sampling immediately after drug administration. The second half-life reported by Huffman et al. (1973) corresponds well with the first phase half-life of Stoller et al. (1975) and Pratt et al. (1974) and is between 2.0 and 4.0 hours. This half-life is attributed to renal clearance (Bleyer, 1977). The third and final phase half-life, attributable to biliary recycling, has been reported as 10.4 ± 1.8 hours by Stoller et al. (1975) and as 26.99 ± 4.44 hours by Huffman et al. (1973). The discrepancy in the terminal phase half-life may be due either to differences in dosing schedules, or, the assay method used to measure MTX (Stoller et al. 1975). The third phase has been implicated in the toxicity associated with high dose-MTX regimens (Djerassi, 1975). These observations suggest that the toxic effect of MTX on normal tissue is primarily a function of the duration of exposure to suprathreshold concentration of of drug, rather than the peak plasma levels which may be achieved (Bleyer, 1977).

Huffman et al. (1973) calculated two volumes of distribution for MTX: an initial distribution volume was reported as 13.8 ± 1.09 L. which is considerably larger than the plasma volume and indicates rapid tissue distribution of MTX. The second value reported was the apparent volume of distribution at steady state which was

73.3 ± 15.6 liters.

Methotrexate has been shown to be protein bound, primarily to serum albumin. Wan et al. (1974) reported a mean of 45% of plasma MTX bound to protein in twelve patients with drug concentrations between 0.3 and 3 mg. ml.$^{-1}$. Liegler et al. (1973) determined MTX plasma protein binding to be between 50 and 70% in a group of fifteen patients. Taylor and Halprin (1977) also found approximately 60% of serum MTX bound to albumin. In a more recent publication, Steele et al. (1979) reported MTX binding to albumin to be approximately 87% and to total plasma protein; 95% over a concentration range of 1 to 30 micromoles l^{-1}. These authors also performed Scatchard analysis on their data and determined that MTX binds to two distinct classes of binding sites (Steele et al., 1979). The displacement of MTX from its protein binding site(s) has been demonstrated with both para-aminohippuric acid (Liegler et al., 1970) and salicylic acid (Taylor and Halprin, 1977).

Methotrexate, widely distributed in body tissues, is concentrated in the liver; liver to plasma ratios of 8:1 24 hours after intravenous administration of 80 mg m^{-2} have been reported (Creaven et al., 1973). Dedrick et al. (1973) reported, for the rat, tissue:plasma partition coefficients of 3 and 1 for the liver and skin respectively. Distribution into interstitial fluid spaces; cerebrospinal fluid, pleural, and peritoneal cavity occurs

slowly, with characteristics described by Bleyer (1973) as
resembling a   passive transport system. Transport across
cell membranes is achieved via an energy-coupled carrier
mediated mechanism which has been described in detail
(Flament-Durant, 1975 and Hall et al., 1966). One of the
reasons for employing high dose-MTX regimens is to produce
plasma drug concentrations sufficiently heigh to permit
facilitated diffusion into tumor cells deficient in
transport sites (Bender et al., 1977).

iii. Metabolism

Early studies of MTX administered at "conventional"
doses suggested that, in man, the drug is excreted
unchanged into the urine (Henderson et al., 1973). Wan et
al. (1974) detected a metabolite of MTX excreted in the
urine which accounted for approximately 6% of a 30 mg. m^{-2}
intravenous dose. Following oral administration of the
same dose, 35% of the drug was excreted in the form of
metabolites (Wan et al., 1977). It has also been shown
that MTX is metabolized during enterohepatic circulation by
bacterial organisms within the gastrointestinal tract
(Bleyer, 1977). These reports suggest that MTX metabolism
in man occurs primarily in the gastrointestinal tract or
"enterohepatic circuit" (Bleyer, 1977). Jacobs et al.
(1976) discovered that humans excrete "significant" amounts
of 7-hydroxymethotrexate when doses of MTX greater than 50
mg Kg.$^{-1}$ are administered intravenously. The discovery of

this metabolite is significant because of its extremely low aqueous solubility which has been implicated as the cause of renal toxicity often associated with high dose MTX regimens (Jacobs et al., 1976).

iv. Excretion

Excretion of MTX is primarily renal. Huffman et al. (1973) reported that ninety-six hours following intravenously administered MTX, 66% to 81% of the administered dose was excreted unchanged. The remaining 19% to 34% was accounted for by various metabolites. Huffman also reported a renal clearance rate of 78 $\pm$ 4.9 ml. min.$^{-1}$ for "low" plasma concentrations of MTX. This report indicates that MTX is reabsorbed since the renal clearance was less than the glomerular filtration rate in the patients studied. The clearance of MTX at "high" plasma concentrations was significantly higher than inulin clearance in 13 patients, suggesting active secretion of the compound (Liegler et al., 1970). Approximately 1.5% of an intravenous dose appears in the feces, most likely caused by excretion of MTX from the liver into the bile (Huffman et al., 1973). These authors also reported that some patients showed two or three increases in the rate of MTX excretion during a 96 hour urine collection period. Since these increase were associated with ingestion of food, it was suggested that MTX undergoes substantial biliary secretion and subsequent reabsorption (Huffman et

al., 1973).

e. Pharmacokinetic Models

The use of mathematic models in relation to drug disposition is important in formulating generally applicable predictions of plasma concentrations before drug is administered. In an effort to predict MTX plasma levels in man, several models describing its disposition have been developed.

Stoller et al. (1975) used a two compartment open model, based on Equation 1 to describe MTX.

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

(Equation 1)

Huffman et al. (1973) found that plasma concentration-time data could best be described by a three compartment open model and the corresponding tri-exponential equation.

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

(Equation 2)

Reich (1977) has developed an expanded model consisting of seven distinct compartments including shallow and deep intracellular spaces, metabolite space, metabolite extracellular space and urine. An interesting aspect of this model is the inclusion of Michealis-Menton kinetics to describe appearance of drug in the urine, however, the

advantage of such a complex model over the previously described, simpler models has yet to be demonstrated.

Bischoff et al. (1970,1971) have developed a "physiologic-perfusion" model to characterize the dispostion of MTX. Contrary to compartments with little or no physiologic meaning, the perfusion model is composed of specific body regions with similar physiologic characteristics such as blood flow, and drug:tissue partition coefficients. The significance of perfusion models is in their ability to reflect physiologic conditions which affect drug dispostion whereas lumped compartments are not suitably flexible for this purpose.

6. Leucovorin Disposition

Although the general disposition of orally or parenterally administered LEU has been determined, there have been no published reports which quantitatively characterize its pharmacokinetics, either alone or in combination with high-dose MTX therapy (Nixon, 1979). The majority of published data which include LEU'S disposition, is primarily involved with its rate of conversion to METHF, the major component of naturally occuring, circulating serum folates (Herbert et al., 1962). Furthermore, much of these data are of a qualitative, rather than a quantitative nature, consequently, pharmacokinetic interpretation of these works is not possible.

a. Plasma

i. Intravenous

Spray and Witts (1953) were the first to examine, in humans, the rate of disappearance of intravenously administered LEU in normal subjects and patients suffering from "anemia". Following injection of 1.0 mg, peak plasma levels measured 10 minutes after dosing, varied from 44 to 76 ng ml^{-1} in normal subjects, and 33 and 54 ng ml^{-1} in anemic patients. The authors made no statistical inferences regarding this data. The rate of disappearance of LEU was described as "rapid", plasma levels were less than 10% of maximum within 1 1/2 to 2 hours after dosing. No differences in elimination rate between normal and anemic subjects was noted. These authors reported a "folinic acid-space" which was calculated by dividing the total amount of LEU in the body by the corresponding plasma concentration. Two such spaces were reported; 10 minutes and 90 minutes post drug administration. At 10 minutes, the value of this space had a mean value of 17.2 liters in normals and 24.2 liters in anemia patients, which demonstrated a statistically significant more rapid uptake in the anemia patients. The space calculated at 90 minutes for controls and anemia patients was 342 liters and 547 liters respectively. However, the meaning of this space is unclear, since it may reflect either the conversion of LEU to other folate forms or, that LEU is quickly taken up by

tissues. A representative plot of drug concentration as a function of time from the normal subjects is shown in Figure 4. Baker et al., (1965), characterized the fate of parenterally administered "folinic acid" (LEU) by determining plasma folate activity from P. cerevisiae growth. Because a "great increase" in this organism's growth was observed, these authors concluded that "parenterally administered folinic acid circulates unaltered".

The disposition following intravenous administration of 5 ug Kg^{-1} of double radiolabelled folinic acid (5-formyl-^{14}C-FH4-^{3}H), in a human patient was described by Nixon and Bertino (1972). Serum collected 90 minutes after dosing revealed that 40% of the radiolabel co-chromatographed with a folinic acid marker. Sixty percent of ^{3}H label was present as METHF, demonstrating conversion of LEU to the predominant circulating folate. The remaining markers had been "labilized to non-absorbable materials, perhaps amino acids" suggesting that little LEU remained in the circulation ninety minutes after dosing.

In a more recent study, Rothenberg et al., (1979), characterized the plasma clearance of LEU following rapid intravenous injection in two normal subjects. The radiochemical assay measured both the active and inactive diastereoisomers of LEU. Peak plasma concentrations achieved in those two subjects following doses of 6.0 mg. and 3.0 mg. were approximately 1100 ng ml^{-1} and 1000 ng
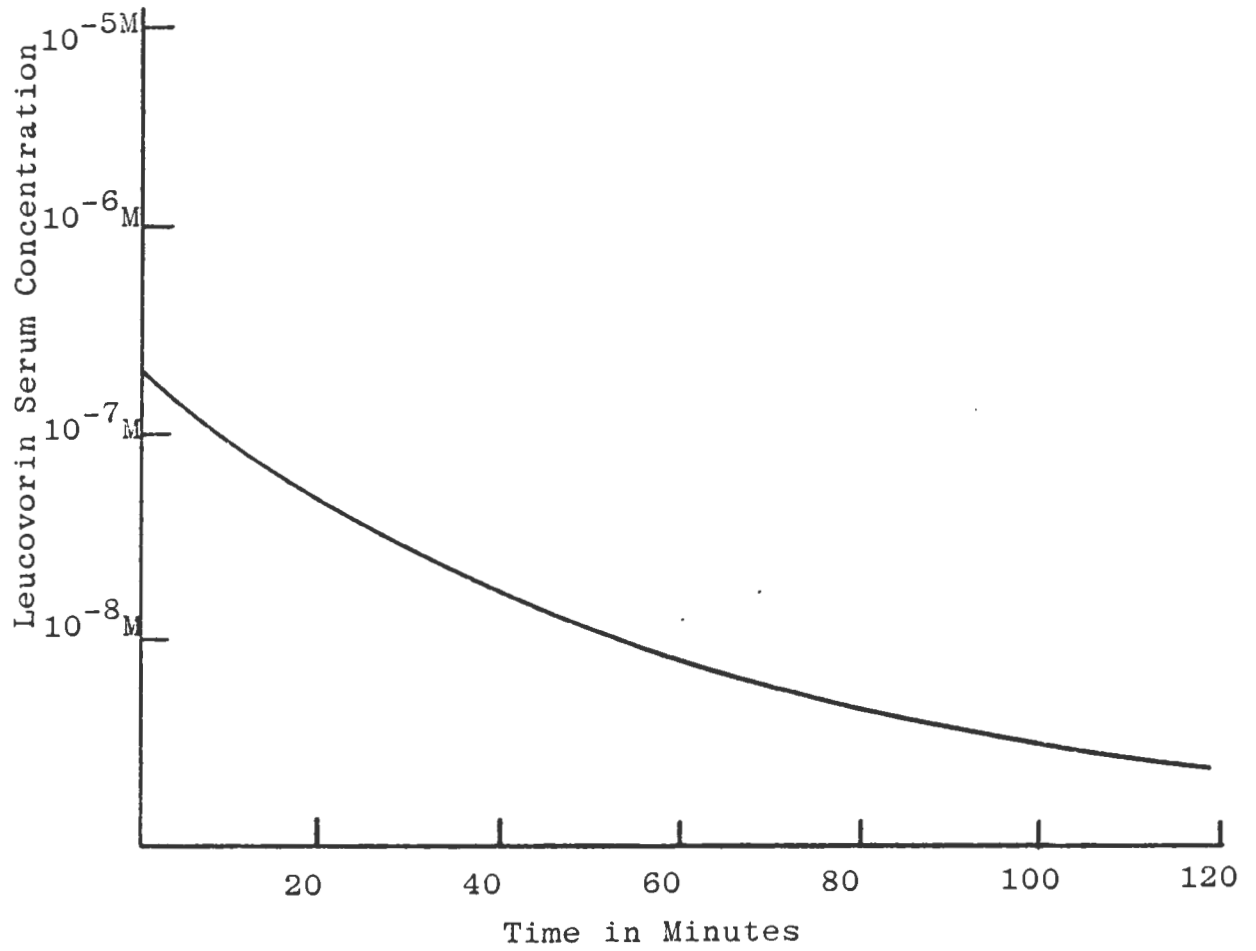
Figure 4: Leucovorin Plasma Decay as a Function of Time as Measured by a Microbiologic Assay Method

ml⁻ᵗ respectively. The plasma profiles showed a "rapid clearance for the first 30 minutes" which "then plateaued and cleared very slowly over the next 90 minutes". The description of a plateau following the rapid initial decline in plasma LEU is contrary to the reports of Spray and Witts (1953) and Nixon and Bertino (1972) who described only a rapid plasma clearance with no mention of a second elimination phase. Rothenberg et al., (1979) suggest the slow second phase clearance is most probably due to the accumulation of the inactive diastereoisomer, since the kidney appears to preferentially excrete only the active form of LEU.

Perry and Chanarin (1970), Chanarin and Perry (1970), and McLean (1967), have performed a series of experiments designed to determine the origin of serum METHF after the intravenous administration of tritium labelled folate analogues. These authors reported that "when given parenterally...these compounds exchange with folates in body tissues, that is, when tritium-labelled dihydrofolate or tetrahydrofolate...is given by injection the labelled folate disappears from plasma and is replaced by an unlabelled form...". Furthermore, Johns et al., (1961) reported that "when a parenteral dose of tritium-labelled folic acid in man was followed by a large dose of non-radioactive folic acid 24 hours later, a large amount of radioactivity was displaced...".

These data suggest that the appearance of METHF in

plasma following the intravenous administration of LEU is caused by a flushing of METHF from tissue rather than a direct metabolic conversion.

ii. Oral

The fate of orally administered folates, including LEU has received considerable attention. Specifically, the rate at which folic acid crosses from the gut into the circulation and the rate and extent to which folates are converted to METHF, the predominant circulating reduced folate analog (Denko,1951; Spray and Witts, 1952; Chanarin, Anderson, and Mollin, 1958). In addition Blakely (1969) has published an excellent review of folic acid biochemistry which includes the disposition of orally administered folate. Although the fate of orally administered LEU has received considerably less attention, interest in this route of administration in MTX-rescue regimens has prompted relatively recent interest in its disposition.

Baker et al., (1965) administered 10mg. of racemic folinic acid to 48 normal subjects. Assay of serum folates was carried out by a combination of TLC and the standard microbiologic assay technique of Sauberlich and Baumann (1948). Serum folate activity (L. Casei) indicated a slow rate of absorption, the peak activity was not reached until 4 hours after administration, followed by a rapid decline in activity at 8 hours, the last sampling time. Because

little or no activity was noted for either S. faecalis or P. Cerevisiae, it was concluded that the circulating folate was not LEU but rather METHF which was rapidly converted from LEU in the intestine.

Perry and Chanarin (1970) noted that, with the exception of Baker et al., (1965), almost all previous reports concerning the intestinal absorption of folates dealt with folic acid alone. These authors reported the absorption of the reduced folate DHF and THF as well as their formyl and methyl derivatives. The evidence presented in this paper suggest that DHF is first reduced to THF and then methylated in the small gut during its intestinal absorption. Furthermore, "...tetrahydrofolate which is already reduced is methylated". It is suggested that the enzyme dihydrofolate reductase is involved with these reductions in the small intestine. The selectivity of this reduction is demonstrated by the rapid conversion of DHF to THF compared to the reduction of folic acid to DHF which is reported to occur at approximately one-tenth the rate of the former reaction (Zakrewski and Nicol, 1960). Thus, it was reported that the reduced forms of folate, including LEU, were completely reduced to THF and methylated, whereas folic acid was only partially reduced.

Whithead et al., (1972) described the intestinal conversion of 2 mg. of racemic LEU to METHF in 3 subjects. Using the standard microbiologic technique, folate activity was measured in both the peripheral venous plasma and the

hepatic portal venous plasma. These authors defined newly absorbed folate as "the difference between the increment of folate activity in hepatic portal vein plasma and in systemic plasma". Although the data is not suitable for pharmacokinetic analysis certain trends are evident. Peak folate activity was reached between 30 and 60 minutes of administration in all 3 subjects. However, the rate of drug clearance among the subjects varied greatly. The half-life was estimated as 45 minutes in one subject, 15 minutes in the second, and in the third a plateau in plasma concentration was observed making an estimate of half-life impossible. In agreement with Perry and Chanarin (1970), the predominant circulating folate found in plasma was not LEU but rather, METHF, indicated by an increased L. casei activity and little activity for either P. cerevisiae or S. faecalis. A small amount of plasma folate active for these organisms was found early in the course of absorption. Whithead et al., (1972) suggest that a small amount of folinic acid may have reached the plasma via passive absorption with water through the intracellular spaces. The appearance in plasma of METHF, measured by L. casei activity, following LEU administration was also observed by Pratt and Cooper (1971). However, S. faecalis and P. cerevisiae activity was seen in plasma 30 minutes after dosing, suggesting that some LEU had been absorbed. Thin layer chromatography of serum samples confirmed that this folate was indeed LEU (Pratt and Cooper, 1971). The

absence of an increase in P. cerevisiae activity in the bile was taken by these authors to mean that little if any LEU appeared in the bile. Rather, it is concluded that folates appearing in bile reflect the "intracellular folates of liver", and not orally absorbed analogs.

LEU is known to form, under the acid conditions existing in the stomach, N^5-N^{10} methenyl THF which in turn forms, in the basic environment of the small intestine, the readily oxidized and unstable N^{10} formyl THF (May and Bardos et al., 1951). Because the suitability of N^{10} formyl THF for rescue following high dose-MTX has not been established, LEU has been used parenterally in rescue protocols (Nixon and Bertino, 1972). Nixon and Bertino (1972) used radiotracer techniques to determine whether orally administered LEU reached the blood pool in a form that "contributes to expand the body pool of reduced folate coenzymes and so potentially to reverse the effects of antifolates". At doses of this compound as high as 50 ug Kg^{-1}, oral absorption was "close to 90 percent", since only 8% of the ^3H and 5% of the ^{14}C was excreted in the stool within five days of drug administration. During transfer from the gastrointestinal tract to the systemic circulation substantial metabolism of LEU occured. Ninety percent of the serum folate^3-H was identified as labelled METHF, while that label associated with the ^{14}C-fORMYL group did not appear among the circulating serum folates, suggesting that this portion had been taken up by tissues.

These data taken with the previously described work of Whithead et al., (1972), Perry and Chanarin (1970, and Baker (1965), demonstrate that orally administered LEU raises levels of serum folate activity for L. casei but not S. faecalis or P. cerevisiae, and identifies METHF as the predominant circulating folate. Because METHF can circumvent the enzyme block imposed by MTX, these works suggest that orally administered LEU may be useful in high dose-MTX regimens.

Mehta et al., (1978) recently investigated the serum distribution of LEU following oral administration of 15 mg. of drug as a solution and as a tablet. Within 30 minutes, of oral administration, 90% of the administered folate was assayed as METHF. Peak plasma levels of LEU were observed 30 minutes following dosing and fell off rapidly afterwards. The plasma half-life of LEU was reported as 0.64 ± 0.07 hr. and 0.67 ± 0.07 hr after oral liquid and oral tablet administration respectively. From these data, it was further observed that peak METHF levels were achieved approximately 2 hours following the administration of drug. METHF was determined to have a half-life of approximately 2.2 hours.

The reported LEU half-life of 0.67 hr is in general agreement with the values of 30 minutes reported for the elimination phase half-life determined by Spray and Witts (1953). The fact that Mehta et al., (1978) did not describe a distribution half-life is most likely caused by

difficulties in detecting rapid distribution following oral drug administration (Wagner, 1975).

b. Urinary Excretion Data

The renal excretion of folic acid has received considerable attention (Mclean and Chanarin, 1966; Goresky et al., 1963; Chanarin and Mclean, 1967), and has been reviewed by Blakely (1969). METHF, N^{10}-formyl THF and N^5,N^{10}-methenyl FH4 are the most commonly detected urinary folates. There is however, a paucity of data dealing specifically with urinary excretion of LEU.

Spray and Witts (1953) followed the urinary excretion of LEU following the intravenous administration of 1 mg. of drug to 5 normal subjects. The percent of drug excreted during the first two hours after dosing ranged from 9.9 to 19.2, and had an average value of 15.4. No folate activity attributable to LEU was observed after 2 hours, indicating either conversion to another form or uptake of the drug by various tissues.

Nixon and Bertino (1972) found a "fairly-constant" rate of urinary clearance of N^{10}-formyl and N^5,N^{10} methenyl FH4 following the administration of double radio-labelled LEU. In addition, these authors reported that the portion of urinary METHF was approximately proportional to the serum concentration of identifiable radio-labelled METHF. Furthermore, "large amounts of radio-labelled 5-formyl" FH4 were found in the urine 0-2 hours post dosing, even when no

corresponding label was identifiable in the serum. In a similar study of administered radio-labelled METHF, no formyl folate was excreted in the urine (Nixon and Bertino 1970). These data led the authors to conclude that the "...kidney may have both regulatory and metabolic roles in the excretion of folates: serum 5-methyl FH4 may be conserved by the kidney in preference to 5-formyl FH4...".

Recently, Rothenberg et al., (1979) examined the urinary clearance of LEU in a subject who received 3 mg. of intravenously administered racemic LEU. The total urinary excretion in two hours was calculated to be 578 ug or 19.3% of the administered dose, a value that is in general agreement with the earlier work of Spray and Witts (1953). The urinary excretion of LEU in two subjects receiving only the active isomer of LEU was also determined. The two hour cumulative urinary excretion in the 2 subjects were 16.6% and 20% of the administered dose. It was concluded from these data that since the percent of dose excreted following the administration of either the racemic mixture, or active diastereoisomer, of LEU were similar that only the active rather than the inactive form is rapidly excreted.

C. 5- Methyl Tetrahydrofolate

1. Physical-Chemical Properties

Prefolic A (5-methyl THF, METHF), N-amino-5- methyl 5,6,7,8,-tetrahydro- 4-hydroxy-6-pteridinyl)methyl

aminobenzoyl L-glutamic acid, is a yellowish white or white crystalline powder. The anhydrous barium salt has a molecular weight of 594.8, commercial preparations usually contain four molecules of water per mole of compound (Sigma Product Catalog, 1980). The aqueous solubility of METHF has been reported as 1.1 grams per 100 ml. It is practically insoluble in organic solvents (Conti et al., 1974). The melting point of METHF is reported as greater than 300°C. (Conti et al., 1974). At alkaline pH, METHF is easily oxidized by air or hydrogen peroxide-perioxidase to a dihydrofolate (Gupta and Huennekens, 1967), which in turn, can be reduced back to METHF by hydrogen in the presence of platinum, ascorbic acid or borohydride (Blakely, 1969).

Spectral characteristics for METHF, in phosphate buffer pH 7.0, have been reported (Blair and Saunders, 1977; Conti et al., 1974; and Chanarin and Perry, 1967). The molar absorptivity was determined to be 3.18×10^{-4} m^{-1} cm^{-1} (24a). The ultraviolet spectrum showed a maximum at 290 nm. and a minimum at 245 nm. (Conti et al., 1974, and Chanarin and Perry, 1967). The infrared spectrum for the magnesium salt has ben characterized by Conti et al., (1974), and the proton magnetic resonance spectrum was reported by Blair and Saunders (1970).

2. METHF, Isolation and Identification

Donaldson and Keresztesy (1959) reported the

extraction from horse liver of a "citrovorum factor
precursor" which did not support the growth of either S.
faecalis or L. citrovorum. The precursor, named Prefolic
A, was reportedly converted to citrovorum factor by two
separate enzyme systems, an FAD-Linked system and a
transformylase mechanism (Donaldson and Keresztesy, 1959).
These authors also showed that Prefolic A could be readily
converted, via enzymatic oxidation, to THFA and
formaldehyde.

Larrabee and Buchanan (1961) discovered an
intermediate of methionine biosynthesis which was
identified as a folate compound containing a one carbon
substituent. The folate derivative did not support the
growth of either S. faecalis or L..ul. citrovorum, but it
was later determined that it would support the growth of L.
casei. (Larrabbe et al., 1961). This compound was found to
contain approximately one mole of methyl group per mole of
compound. Larabee et al. (1961) used Prefolic A as the
substrate in the conversion of methionine to homocysteine
(in the presence of DPNH, FAD, Mg^{+2}, ATP and "B12 enzyme").
These authors proposed N^5 methyl THF as the tentative
structure of the folate derivative (Larrabbe et al., 1961).

Prefolic A, as the barium salt, was isolated in
"essentially" pure form from horse liver by Donaldson and
Keresztesy (1961) who reported that its spectral
characteristics were similar to those of dihydro and
tetrahydro folic acids. Keresztesy and Donaldson (1961)

reported the chemical synthesis of Prefolic A from dl THF. The synthetic material was approximately as active as the natural Prefolic A relative to L. casei activity, and it was found to have the same ultraviolet absorption characteristics (Donaldson and Keresztesy, 1961). Blair and Saunders (1974) have since reported the preparation of (+,-)-METHF from folic acid, using sodium borohydride as a reducing agent.

3. Biochemical Role of METHF

The enzyme N^{5-10} methylene THF reductase, found in the liver of various vertebrates and in certain bacteria (Blakely, 1969), catalyses the reduction of N^{5-10} methylene THF to METHF. This reaction represents the major biosynthetic pathway for METHF. This reaction can use either NADH, NADPH, or $FADH_2$ as the electron donor (Donaldson and Keresztesy 1962).

Wagner (1966) isolated an enzyme, tentatively named trimethylsulfonium: tetrahydrofolate methyltransferase which catalyzes the methylation of THF as shown in Figure 4. This, however, is not a major mechanism for the synthesis of METHF.

In addition to the identified enzyme mediated mechanisms of METHF biosynthesis, indirect evidence suggests a third pathway. Serum concentrations of METHF are much higher following oral administration of folic acid or folinic acid (LEU) than after identical doses

administered parenterally (Nixon and Bertino, 1972, Mehta et al., 1978). This observation has led to speculation that significant synthesis of METHF occurs in the intestinal mucosa (Baker et al., 1965, and Chanarin and Perry, 1970) although the precise mechanisms responsible for the synthesis have not yet been elucidated.

Larabee et al. (1961) first identified METHF as the folate intermediate in the interconversion of homocysteine to methionine. In the presence of appropriate enzymes and cofactors METHF transmethylates with homocysteine resulting in the synthesis of methionine and THF.

This transmethylation requires several cofactors: ATP, Mg^{+2}, and either FADH2, NADH or FAD (Larrabbe et al., 1961) as the electron donor. This appears to be the only synthetic pathway involving METHF.

III. FOLATE ASSAYS

A. Microbiologic

Interest in the quantification of serum folate concentrations was initially focused on determining the etiology of various anemias (Kamen and Caston, 1974), by microbiologic methods. Sauberlich and Baumann (1948) described such an assay using Leuconostoc citrovorum ATCC 8081, later identified as Pediococcus cerevisiae, for which LEU is an essential growth factor. The determination of LEU concentration (or activity) was carried out by measuring the zone of growth around standard paper discs saturated with leucovorin solutions. Lactobacillus casei ATCC 7469 and Streptococcus faecalis 9 have been used in a similar manner (Grossowicz et al., 1962).

The microbiologic assays are not specific. For example, L. casei ATCC 7469 responds not only to pteroyl glutamic acid but LEU and also certain polyglutamates (Grossowicz et al., 1962). S. faecalis utilizes folic acid and several tetrahydrofolic acid derivatives as does P. cerevisae. In addition to their lack of specificity, microbiologic assays are generally time consuming, often requiring eighteen to twenty-four hours of incubation time and, more importantly, may be affected by antimicrobial and antineoplastic agents in the blood (Kamen and Caston, 1974). However, Hutchinson and Burchenal (1952) isolated a methotrexate resistant strain of L. citrovorum ATCC 8051

and Mehta and Hutchinson (1975) reported the use of this strain for the determination of LEU in the presence of methotrexate. The eventual isolation of a methotrexate-resistant strains of L. casei and S. faecalis made possible the determination of METHF as well (Mehta and Hutchinson, 1977). L. casei responds to all forms of folate, from which total folate activity can be measured, while S. faecalis var. Durans responds to all forms of folate except METHF (Mehta and Hutchinson, 1977). Thereby, the difference in activities between the two microbiologic assays is attributable to serum levels of METHF. These methods, although useful, remain subject to interference by antimicrobial agents and other antineoplastic agents which may be used in combination chemotherapy.

B. Spectrophotometric Methods

1. Ultraviolet

The extinction coefficient (282 nm.) for LEU has been reported as 2.39 to $2.41 \times 10 M^{-1}$ cm^{-1} in 0.1N NaOH (Zakrzewski and Sanson, 1971). Ultraviolet spectroscopy, however, has not been employed for LEU analysis for two reasons: (1) the molar absorptivity reported is not considered to be reliable because of frequent impurities in samples; and (2) LEU readily dehydrates under acidic conditions to N^5,N^{10} methenyl THF, which absorbs at 352 nm., thus affecting the accuracy of leucovorin analysis

(Ponte et al., 1979).

Pont et al., (1979) state that LEU may be analyzed by acidification with 0.1 N HCl followed after 1.5 to 2.0 hours by UV measurement, provided that no interfering species are present. Interfering species are considered to be folate derivatives which might convert to anhydroleucovorin upon acidification. Because such folates are thought to exist in blood (Blakely, 1969) UV spectroscopy is not considered applicable to the analysis of LEU in biologic fluids.

2. Fluorometric

LEU exhibits maximum natural fluorescence at pH 7.0 with excitation and emission at 370 nm and 460 nm (Dugan et al., 1957) and 314 nm and 365 nm (Uyeda and Rabinowitz, 1963) respectively. The differences in wavelengths reported were attributed to sample impurities, pH variations, and quenching (Ponte et al., 1979). Although LEU will fluoresce and quantitative results are similar to those obtained by microbiologic methods (Ponte et al., 1979), fluorometric methods have not been applied to the determination of LEU.

3. Other

Several radiochemical techniques for serum folate analysis have been reported recently (Kamen and Caston, 1974; Rothenberg et al., 1979; and Raso, 1977). All of

these methods rely on the competition between METHF and tritiated pteroylglutamic acid (^{3}H PGA), for the binding sites on naturally occuring protein. (METHF is used as the reference folate because it is the major circulating serum folate.) The protein source may vary but milk protein is the most common binder in use (Rothenberg et al., 1979). In these methods, tritiated ^{3}H PGA is displaced from the binding site by METHF, and standard curves are prepared by plotting percent ^{3}H PGA bound as a function of METHF concentration. The higher the METHF concentration, the less ^{3}H PGA bound.

These techniques, however, cannot be applied to LEU because the affinity of the binding protein for the formyl tetrahydrofolate (LEU) is not strong enough to displace ^{3}H PGA (Rothenberg, et al., 1979). Further, since some circulating folate is present in the bound form these methods do not reflect total folate activity but rather are an indication of activity relative to METHF standard (Kamen and Caston, 1974). Finally, the effect of other agents notably antimicrobials and antineoplastic agents (e.g., methotrexate) on these methods has not been reported.

Rothenberg et al., (1979) developed an indirect approach in order to measure LEU using the radiochemical technique. METHF is first measured in a portion of serum or urine sample using standard radiochemical techniques. Another portion of the same serum or urine sample is then acidified and exposed to sodium borohydride which converts

LEU (formylPH4) to methylPH4. The difference in METHF concentrations between the treated and untreated samples is assumed to be due to the original concentration of LEU.

Langone and Levine (1979) have published a preliminary report of a radioimmunologic assay for leucovorin using an anti-leucovorin antibody produced in rabbits. The method is reported to be sensitive to 0.10 nanograms of LEU added to serum. Other forms of folates and methotrexate reportedly do not interfere with the determination of LEU; however, due to the antibody's specificity, the method is not applicable to the predominant circulating folate, METHF.

Anion exchange chromatography was the first HPLC application for the separation of mono-pteroylglutamates (Stout et al., 1976 including LEU and METHF (Reed and Archer 1976). However, these methods are relatively inefficient; in addition they require long chromatographic columns (4 1/2 ft.) and unacceptably long chromatographic run times. Other HPLC methods have recently been reported (Reif et al., 1977, and Chapman et al., 1978) and a complete review of chromatographic mmethods applicable to various pteridine and/or pteroylglutamates has been published (Montgomery et al., 1975).

The use of HPLC coupled with electrochemical detection has been recently reported for the determination of METHF in plasma and spinal fluid (Lankelma and Van der Kleijn, 1980). A detection limit of $2 \times 10^{-9} M$ METHF and 100%

recovery from body fluids is claimed. The low limit of detection is attributable to on-column concentration of the folate. Sample treatment requires the use of ten percent trichloroacetic acid in 0.1 N HCl to precipitate plasma protein prior to centrifugation and sample injection. Because of "early plasma peaks in the chromatogram" and "foreign eluent", it was necessary to "vent" the column to prevent "poisoning" of the electrode surface (Lankelma and Van der Kleijn, 1980). The method is not applicable to LEU which is not detected under the chromatographic and electrochemical conditions employed. The use of trichloroacetic acid in 0.1 N HCl raises considerable doubt regarding the integrity of the various forms of reduced folate in the samples so treated. For example, when LEU is acidified it loses water to become N^5,N^{10} methenylFH4 (1969). Other reduced folates are subject to rapid hydrolysis as well (Blakely 1969). The possibility exists that all folate present in plasma samples treated by this method are converted to some intermediate form of reduced folate or to METHF. If this were indeed the case the reported sensitivity would be erroneous since the total peak height or peak area would not be due solely to METHF.

In addition to the methods applicable to the assay of LEU and/or for METHF in biologic fluids, several techniques have been reported for the separation and identification of folate derivatives. These methods are not sufficiently sensitive to the analysis of folates in biologic fluids.

Brown, Davidson, and Scott (1973) reported the separation of pteroylglutamates and related compounds by thin layer chromatography. This and other methods have been thoroughly reviewed by Blakely (1960).

IV. ELECTROCHEMICAL DETECTION

A severe limitation to the application of high pressure liquid chromatography (HPLC) to the analysis of drugs in biological fluids has been the lack of a suitable detector (Riggin et al., 1975). Five basic requirements of an HPLC detector have been recently enumerated by Hashimoto and Maruyama (1978): (1) high sensitivity and reproducibility; (2) versatility; (3) continous monitoring of column effluent; (4) independence from operating parameters such as flow rate and mobile phase composition; and (5) a wide linear response. Also important is the ease of operation as well as the durability and stability of the system. The spectrophotometric methods, i.e., ultraviolet, visible and fluorometric, although suitable in many respects, are not applicable for compounds without chromophores, those with low molar absorptivity, or those that do not fluoresce. Refractive index, although versatile, is not particularly sensitive and is therefore not generally applicable for the analysis of drugs in biological fluids.

The possibility of using electrochemical techniques to

monitor HPLC effluents was first discussed by Kemula (1952), although the technology available at that time severely restricted the application of this method. Kissinger et al., (1973), Takata and Muto (1973) and Fleet and Little (1975), have recently described electrochemical detectors compatible with modern HPLC technology. Since these reports appeared electrochemical techniques have been applied widely to the quantification of drug substances in biological fluids (Greenberg and Mayer, 1979; White, 1979; and Munson et al., 1978). Although the electrochemical detector is not the widely sought "universal" detector for HPLC, it provides a selective and sensitive method of detection for suitable compounds.

A. Theory

The coupling of electrochemical detection to high pressure liquid chromatography relies on introducing the column effluent to a region, or cell, in which electrolysis can occur (Takata and Moto, 1973). Electrochemical detectors in current use are based on "DC hydrodynamic chronoamperometry" in which "current is measured as a function of time with a constant potential applied at a fixed electrode exposed to a moving fluid" (Shoup, 1979). This may best be conceived as electrolysis at a fixed point along a flowing stream. Electrolysis can result in either oxidation or reduction reactions. Since reduction of dissolved oxygen, metal ions, and hydrogen can hinder

electrochemical detection, most practical applications have been carried out in the oxidation mode (Shoup, 1979).

Scheme I represents the simplified reaction which occurs when an organic compound R is oxidized to an intermediate compound O, yielding n electrons (e). The unstable intermediate may be expected to form, irreversibly, the stable product P.

$$R \longrightarrow O \longrightarrow P$$
$$e^- \longrightarrow E \qquad \text{(Scheme 1)}$$

This is a simplified view and generally the electrochemistry of organic molecules of clinical significance is not well understood (Kissinger et al., 1979).

The quantity of electricity required for this reaction can be calculated according to Faraday's Law Equation 3.

$$Q = n * F * N \qquad \text{(Equation 3)}$$

Where;

    Q = the number of coulombs used in the conversion
    N = the number of moles of material converted
    n = the number of electrons generated per mole of
        material converted
    F = Faraday's constant

Current is a measure of the rate at which the oxidation proceeds and is described by Equation 4.

$$i = dQ/dt \quad \text{and} \quad nF * (dN/dt)$$

(Equation 4)

Where i represents current and all other terms are as described earlier.

The efficiency with which material is oxidized (or reduced) and current is produced, can be calculated from the number of moles of material that react (N) and the total number of moles entering the electrode region (Ntotal), according to Equation 5.

$$\text{Efficiency (Eff)} = (N * N_{total}) \times 100$$

(Equation 5)

Amperometric detectors typically operate with efficiencies ranging from four to ten percent (Kissinger, 1977). The current produced via electrochemical reactions and hence the efficiency of the system is affected by several parameters, some of which are governed by the electrochemical process and others of which may be controlled experimentally. Equation 6 relates these parameters to the limiting current (i lim)(Willard et al., 1974).

$$i_{lim} = (nFAD \, C_{bulk}) \, / \, \varepsilon$$

(Equation 6)

Where;

n= number of moles of material converted
    electrochemicaly
F = Faraday's constant
A = surface area of the electrode
D = diffusion coefficient of the electroactive species
    in the solvent or mobile phase.
Cbulk = the concentration of the electroactive species
    in the bulk or mobile phase.
(s) = the diffusion layer thickness.

Faraday's constant and D are properties of the
electroactive species and cannot be controlled
experimentally. The diffusion layer thickness and
electrode surface area are variables which can be used to
influence the current generated from the electrolysis of a
sample. Diffusion layer thickness, (s), refers to the
distance through which the electroactive species must
travel from the solution (or mobile phase) to reach the
electrode surface. For an electrochemical reaction to
occur, the electroactive species must: (1) diffuse from the
bulk solution to the electrode surface; and then (2)
transfer electrons at the surface (Kissinger, 1977, and
Willard et al., 1974). The rate-determining step is
considered to be diffusion through the bulk solution, which
is governed by Fick's First Law (Willard et al., 1974),
Equation 7.

$$ J = D * (C_{bulk} - \epsilon) $$

(Equation 7)

Where

J = flux
D = diffusion coefficient
Cbulk = concentration of solute in the bulk phase
Co = concentration in the electrode surface
(s) = diffusion layer thickness

It is apparent from Equations 6 and 7 that flux is inversely proportional to the distance electroactive species must travel and by decreasing (s), the current generated can be increased. Experimentally, (s) can be regulated in two ways: first, by confining the sample within a thin film of electrolyte passing over the electrode surface, an application of the principle of low volume flow thru cells (Figure 5); secondly, by varying the flow rate of solution through the thin layer cell to effect an increase or decrease in the value of (s). Increasing flow compresses (s), resulting in a smaller diffusion distance, thereby increasing $dN \, dt^{-1}$ and the current generated (Kissinger, 1977; and Buchta and Papa, 1976). In practice, however, high effluent flow rates decrease the residence time of electrons within the electrochemical cell and may actually decrease the current generated by an electroactive species.

The rate at which material reaches the electrode surface is not only a function of flux, J, but of the electrode surface area A, (Willard et al., 1974), according to Equation 8.

$$dN/dt = A * J$$

(Equation 8)

Increasing the surface area of an electrode will result in a faster rate of electron production and, according to Equation 8, will generate a larger current. However, the conversion of material in the mobile phase, i.e., electrolytes, trace metals, etc, will also increase. This increase in background current may negate the advantages of using electrodes with large surface areas.

Current may be influenced by another means, not apparent from Equation 6. Equation 9 describes the relationship between the potential applied to the electrode surface and the resulting current:

$$e = e_{\frac{1}{2}} + \frac{rt}{nF} * \ln\left(\frac{i}{i_{lim}}\right)$$

(Equation 9)

Where;
e = applied potential
e1/2 = standard electrode potential
r = molar gas constant
t = absolute temperature
n = number of electrons transferred
F = Faraday's constant
i = current
ilim = limiting current

A current-voltage curve, (hydrodynamic voltammogram) can be produced by plotting current as a function of applied potential. The selectivity of electrochemical detection is based on the difference in behaviour between electroactive species and the currents produced at various potentials. "For example, methoxyhydroxy compounds are

more difficult to oxidize than are catechols and therefore can be 'tuned out' of a chromatogram by lowering the applied potential" (Kissinger, 1977).

B. Electrode Material

A wide range of electrode materials have been applied to electrochemical detectors. Liquid chromatography electrochemical detectors have been prepared from carbon paste (Atuma and Lindquist, 1973), mercury (Fleete and Little, 1974), platinum (MacDonald and Duke, 1973), pyrolytic carbon (Wightman et al., 1978), glassy carbon (Birmingham et al., 1979), silver (Takata and Muto, 1973), and graphite impregnated silicone rubber (Zaradi et al., 1974). Mercury is most often used for reduction reactions while carbon paste and glassy carbon electrodes have been the most widely used for oxidation procedures (Shoup, 1979). Because the reported detection limits for oxidizable substances are considerably lower than for reducible compounds, the various forms of carbon and carbon paste have received the most attention (Wightman et al., 1978).

The choice of electrode material may be governed by several considerations. Mobile phase composition is an important factor; for example, carbon electrodes with oil bases are adversely (in terms of surface distortion) affected to a greater degree by high concentrations of organic in a mobile phase than are either carbon

impregnanted wax or glassy carbon. Many organic compounds react at significantly different rates depending on the electrode used. The greatest sensitivity can be achieved by finding the electrode producing the fastest rate of electron transfer for a given compound (Kissinger, 1977).

Further, the electrode material is prone to auto-oxidation (or reduction) which will contribute to residual (background) current. The extent to which residual currents can be tolerated will also play a role in the selection of the proper electrode.

All carbon paste electrodes suffer from three basic problems: (1) non-reproducibility of electrode surface; (2) adsorption of material from mobile phase; and (3) surface oxide formation (Fleet and Little, 1975). The degree to which electrode surfaces are "poisoned" by the latter two factors, and the ease with which surfaces can be regenerated are paramount in determining the usefulness of an electrode material (Fleet and Little, 1975). A significant disadvantage in the use of electroanalytical techniques is the fact that electrochemical details at any electrode surface are poorly understood, as are the differences between types of carbon electrodes (Kissinger, 1974).

C. Cell Geometry and Operation

Several designs for thin layer, low volume, electrochemical cells have been described (Buchta and Pappa, 1976; Hashimot and Maruyama, 1975; and Riggin et

al., 1975). Virtually all designs use a three electrode configuration (figure 2). The critical parameter in electroanalytical techniques, as discussed by Kissinger (Kissinger, 1977), is accurate control of the potential difference between the electrode and mobile phase (bulk solution). In the normal operating mode the working electrode (w) is set to the desired oxidation potential. The reference electrode monitors the voltage impressed between the working and reference electrode. No current passes through this electrode, it simply measures potential. As the potential at the working electrode drifts, an electronic feedback mechanism adjusts the potential of the auxillary electrode to compensate and return the potential difference between the working electrode and bulk solution to its operator determined value (Kissinger, 1979).

In operation, the carrier electrolyte in the mobile phase will produce a current. This current will increase as the concentration of solute (sample) rises within the electrochemical region. The resistance within the electrochemical cell will drop, and, is compensated for by a decrease in the potential of the auxiliary electrode. This action will result in a constant applied potential at the surface of the working electrode. The change in current produced by the solute (sample) passing through the cell is proportional to concentration as previously described.

V. CHROMATOGRAPHY

"The object of any chromatographic experiment is to separate the components of a mixture by placing a small sample of mixture onto the head of a column of a finely divided partitioning material and subsequently washing or eluting the mixture through the column by passage of a suitable eluent... One hopes that the different components of the mixture will then migrate along the column at different rates and so form discrete bands which disengage as they migrate" (Knox 1977).

Since 1976 an estimated 60% to 70% of the analytical work preformed by High Pressure Liquid Chromatography (HPLC) has been carried out by "reverse-phase chromatography" (Horvath and Melander, 1977). The term "reversed-phase" was coined by Howard and Martin (1950) to distinguish chromatographic systems employing a non-polar stationary phase and a polar mobile phase, from the conventional system of using a polar stationary phase and a less polar mobile or eluent phase (Horvath and Melander, 1977).

In reverse-phase chromatographic systems 5 and 10 um diameter "hydrocarbonaceous bonded" stationary phase particles having octadecyl or octyl functions bound via siloxane bridges to the silica surfaces are the most common (Horvath and Melander, 1978). A wide variety of elution phases such as aqueous buffers, hydro-organic mixtures or

non-aqueous organic solvents may be used, giving the method considerable flexibility. In addition, the use of complexing agents and pH changes can be used to advantage in manipulating retention values.

Reverse-phase chromatography is a form of liquid-liquid chromatography in which the mobile phase is in contact with the stationary phase over a large surface area. Under those conditions, equilibrium distributions of the solute between the two phases occurs rapidly. Non-polar compounds are retained by the stationary phase to a greater degree than are ionic material. Thus, compounds elute from reverse-phase columns in order of decreasing polarity.

The development of ion-pair chromatography has been attributed to Schill and co-workers (Gloor and Johnson, 1977). In this technique ionic or ionizable compounds can be paired with a counter-ion of the opposite charge to form a reversible ion-pair complex. The complex then behaves as an electrically neutral and non-polar compound, which results in the complex having an increased retention in reversed-phase systems. By varying the size or charge of the counter-ion the degree of retention and hence resolution, of a compound can be controlled.

Two mechanisms for the retention of the ion-pair complex have been proposed, the first postulates that the ion-pair complex partitions directly in the stationary phase. The second postulates that the counter-ion

partitions into the stationary phase with the ionic group oriented at the surface, and in this configuration may act as an ion exchange column (Gloor and Johnson 1977). These authors, in a review of practical aspects of reverse-phase ion-pair chromatography, suggest that the actual mechanism is more complex and involves both postulated mechanisms as well as adsorption, micelle formation, and complexation of both the solute and ion-pair reagent. Johnson and Gloor (1977) have published an excellent review of the practical aspects of ion-pair chromatography.

Because most drug substances are weak organic acids or bases, they may be expected to possess some charge, dependent upon pH. Paired-ion chromatography has provided a mechanism wherein the advantages of reverse-phase chromatography: flexibility; selectivity; resolution; and economy can be applied to the analysis of drug substances. The ability to systematically control the separation of ionic compounds of widely different types is perhaps, reversed-phase paired-ion chromatography's most important asset. The application of this technique in drug research, both in quality control and in therapeutic drug monitoring, has been the subject of recent reviews (Johansson et al. 1978, Schill et al. 1977, and Sadee and Beelen, 1980).

VI PROTEIN BINDING

A. Description of Protein Binding Interactions

Protein-drug binding results from an intramolecular interaction between a biological macromolecule and a relatively small drug molecule (Klotz, 1973). The binding of drugs to plasma proteins assumes considerable interest since it is generally accepted that only the free or unbound drug can diffuse across cell membranes, reach receptor sites and exert a pharmacological effect (Ritchell, 1975). Moreover, the extent of protein binding can profoundly affect the distribution and elimination characteristics of a drug (Gillete, 1973 and Dayton, Israili, and Perel, 1973). Competitive displacement from a binding site, by drugs or plasma components, may be an important factor for a drug with a high degree of binding (Wagner, 1975). By increasing the concentration of free drug in plasma, displacement may result in an increase in therapeutic response and lead to drug toxicity. Such is the case, for example, with phenytoin, (Morselli, 1970), dicumarol (Sellers and Koch-Weser, 1970) and MTX (Dixon, 1965). In addition, such alteration in protein binding may affect the rate of elimination, (Gillete, 1973 and Levy and Yacobi, 1974) and alter a drug's volume of distribution. The major plasma and tissue protein responsible for the non-specific binding of most drug substances in albumin (Dayton, et al., 1973 and Chignel, 1971). Other plasma

constituents, eg. the lipoproteins and globulins, may also bind drugs; however, since these components are present in much lower concentrations in plasma than in albumin, their contribution to the protein binding of drugs is considered to be of secondary importance (Spector et al., 1973 and Perkins et al., 1969).

Albumin is composed exclusively of amino acid residues; eighteen amino acids have been identified (Hughes, 1954). The molecular weight of albumin has been reported at between 65,000 and 69,000 daltons (Borga et al., 1969). The concentration of albumin in humans is generally accepted to be 5.9×10^{-4} M, although various disease states can greatly alter the albumin concentration (Dayton et al., 1973). The structure of the protein has been described as an "elongated ellipsoid" with a length and diameter of approximately 150 and 38 Angstroms, respectively (Hughes, 1954). Albumin is a highly charged molecule having about 100 ionizable carboxyl groups and a nearly equal number of groups which are positively charged at the isoelectric point (pH 5.0) (Tanford et al., 1955 and Borga et al., 1969). At physiological pH of 7.4 albumin possesses a net (-19) negative charge. Positively charged drugs will be strongly attracted to the negative sites on the molecule with the formation of strong ionic bonds. Since the macromolecule has a net negative charge, negatively charged drugs are repelled to some extent; however, binding of negatively charged drugs to positively

charged sites can occur (Settle et al., 1971). In addition to ionic bonds, drug-protein complexes can be formed through van der Waal's forces, hydrophobic bonds and hydrogen bonding (Chignell, 1971 and Ritchell, 1975). Basic drugs, positively charged at physiological pH, are loosely bound, occupy a large number of binding sites and are easily displaced. Acidic drugs, negatively charged, are strongly bound to albumin and generally occupy only one or two sites. Displacement of acidic drugs is of a more specific nature than that for basic compounds (Piafsky and Borga, 1977 and Borga et al., 1969). Since acidic compounds bind only to a limited number of sites, displacement occurs only if a second drug has an affinity for the same binding site (Schwartz, 1979). The basic groups from the amino acid residues of arginine, histidine, and lysine and the acidic groups of aspartic acid, glutamic acid and tyrosine are responsible for the binding of acidic and basic drugs respectively (Ritchell, 1975).

B. Equations Describing Protein Binding

Protein binding can be described in terms of the fraction of drug present which is bound to plasma protein. Since, in most cases, the concentration of free drug i.e., the non-protein bound portion (as opposed to bound drug) is determined analytically, the expression regarding the percent of drug bound is frequently written as follows:

$$\text{Percent Bound} = \left[D_t - D_f\right] / \left[D_t\right] * 100\%$$

(Equation 10)

Where Dt and Df represent the total and free drug concentrations respectively.

The degree to which drugs bind to proteins is frequently a function of the total drug concentration present. As the total concentration increases, saturating the available binding sites, the percent of drug bound decreases (Curry, 1970, and Kunin, 1967). Protein concentration also affects binding, low plasma protein levels, such as those in hypoalbuminemia, can reduce the number of available binding sites and result in increased free drug concentration (Dayton et al., 1973 and Lund et al., 1971). Conversely, if the concentration of either albumin or other protein is elevated, an increase in the number of binding sites may result in lower concentrations of free drug (Benhold, 1966). When reporting the extent of drug binding, it is important that the total drug concentration and the albumin (or other binding protein) concentration be described.

Protein binding can also be characterized by using affinity constants and numbers of binding sites to which a drug is bound (Scatchard, 1949). The basic assumption of this method is that all sites of a given class are independent of each other and binding of one molecule does not effect the binding of any other drug molecule.

Drug-protein binding equilibrium is generally

considered to be an instantaneous, reversible process (Meyer and Gutman, 1968). The reaction between drug and protein can be expressed as follows:

$$[D] \quad [P] \quad \rightleftharpoons \quad [DP]$$

(Equation 11)

Where D and P represent the molar concentrations of free drug and protein respectively, DP is the molar concentration of the drug-protein complex, and ka, kd are the association and dissociation rate constants respectively (Scatchard, 1949).

The association constant, Ka, can be written as:

$$Ka = [PD] / [P] [D]$$

(Equation 12)

The degree of binding, r, can be expressed as the moles of drug bound per mole of protein:

$$r = [PD] / [PD] + [P]$$

(Equation 13)

Which can also be defined as:

$$r = Ka [D] / (1 + Ka [D])$$

(Equation 14)

If the protein is determined to have n independent and equivalent binding sites, the quantity r can be written as follows:

$$r = nKa [D] / (1 + Ka [D])$$

<div align="right">(Equation 15)</div>

If more than 1 group of equivalent binding sites are present, Equation 13 can be expressed in a more general form:

$$r = n_i * Ka_i [D] / (1 + Ka_i [D])$$

<div align="right">(Equation 16)</div>

Where ni represents the number of binding sites of class i and Kai is the association constant for class i.

Numerical values for n's (number of binding sites) and Ka's (affinity constants) can be calculated either by graphical techniques or by computer analysis. The Scatchard method (Scatchard, 1949) in which (D) as a function of r is plotted is the most common method by which protein binding data is presented. The Scatchard equation in this form is written as follows:

$$r/a = Ka * N - r*Ka$$

<div align="right">(Equation 17)</div>

When only one type of binding site is present, this plot will yield a straight line with a slope of -Ka and with X and Y intercepts equal to n and nKa respectively. If multiple binding sites are present a plot of this type will become non-linear and is best analyzed by non-linear

iterative techniques. In addition to the Scatchard method, Klotz (1946) and Scott (1956) have presented transformations of Equation 8 that are linear for single site binding systems but become non-linear in the case of multiple binding sites. Although either of these methods will provide protein binding parameters they are not as popular as the Scatchard method.

Several methods describing the computer analysis of protein binding in the case of multiple binding sites have been reported (Metzler et al., 1974; Schwartz, 1979; and Fletcher and Ashbrook, 1973). Each of these methods uses iterative techniques for obtaining estimates of affinity constants and numbers of binding sites.

C. Methods of Studying Protein Binding

Many methods are available for the study of drug binding to proteins. These methods depend on determining the concentration of free drug or detecting a change in a physicochemical property of the bound drug or binding protein due to the drug-protein interaction (Klotz, 1973). Techniques for studying binding can be broadly classified as either spectrophotometric or non-spectrophotometric (Chignell, 1971). The spectrophotometric methods include optical spectroscopy, optical rotatory dispersion, circular dichroism, and fluoresence. (Klotz, 1973). Electron spin resonance, nuclear magnetic resonance and x-ray crystallography, although not strictly spectrophotometric

methods, may be included in this broad classification. Advantages of these methods include the sensitivity attainable and their non-destructive nature.

The non-spectrophotometric methods rely on determining the concentration of unbound drug following the distribution of free and bound forms between two phases (Klotz, 1973). Equilibrium dialysis is the most commonly used non-spectrophotometric technique. Ultrafiltration, ultracentrifugation, diafiltration, and gel filtration are also included in this category.

Ultrafiltration, like equilibrium dialysis and gel filtration, relies on the physical separation of unbound and protein bound drug. (Bush and Alvin, 1973). This separation is accomplished by the use of a membrane which allows unbound drug to pass but retains macromolecules including the drug-protein complex. The membrane used for ultrafiltration usually consists of a microporous cellulose base coated with a continuous polymer film (Chignell, 1977).

In ultrafiltration, a sample containing both drug and protein is placed in a reservoir on one side of a membrane. Unlike dialysis in which the driving force governing the separation is simple diffusion, ultrafiltration relies on nitrogen gas pressure as the driving force for free drug to cross the membrane. The concentration of drug in the filtered aliquot (Df) and in the sample solution (Dt), are measured and the percent drug bound is calculated according

to Equation 1. This process may be repeated to determine binding over a wide range of total drug concentration.

The advantages of ultrafiltration include the speed in which analysis can be achieved and the small sample volume (usually 2-3 ml.) which is required.

D. Folate Binding

1. Folic Acid

The binding of folates to human serum is complex. The extent of binding reported for folates ranges from almost nothing to approximately 65 percent depending on the method used for study.

Metz (1968) reported the binding of folic acid to human serum to be "weak". Condit and Grob (1958) and Neal and Williams (1965) reported that almost no folic acid was bound to plasma proteins when determined via elctrophoresis. Elsborg (1972), however, has suggested that the electrophoretic process might disrupt the drug-protein complex.

Measuring folic acid activity by L. casei activity, following Sephadex chromatography, Markkanen et al. (1972), Markannen and Peltola (1971), and Markannen (1968) determined that approximately 30% to 40% of folic acid activity is bound to plasma protein. Moreover, binding was found to occur with alpha2-macroglobulin, albumin, and transferrin.

Johns and Sperti (1961), using equilibrium dialysis at

37°C, calculated the binding of folic acid to human serum
(determined using tritium labelled pteroylglutamic acid) to
be a constant 64% over a concentration range of 5 to 3000
micrograms/liter. Similar results (70.3%) were reported by
Alter et al. (1971) using the same technique.

Ultrafiltration of plasma samples containing tritiated
folic acid demonstrated 55% to 65% (Neal et al., 1965) and
50% to 55% (Elsborg, 1972) of total folic acid to be bound.

In addition, increased binding capacity for folates
has been shown in patients with uremia, leukemia and folate
deficiency states. Rheumatoid arthritis was found to
result in a decreased folate binding capacity (Alter,
1971).

The extent to which folates bind with plasma proteins
has been reported, the nature of the binding i.e, weak and
nonspecific or "tight" and of a specific nature has been
only recently addressed. Zettner and Duley (1974) studying
folate binding by incubating serum with tritiated PGA,
determined the reaction to be a "reversible equilibrium"
process. They suggested that the large differences
reported in the binding of folate to human sera result from
the folate binders being at varying degrees of saturation.

The specificity of the folate binding site is
suggested by Zettner and Duly (1974) through experiments in
which tritiated PGA, METHF, leucovorin, and methotrexate
(in descending order) were found to be capable of lowering
the binding of folic acid. Non-folate compounds, however,

e.g., barbiturates, phenytoin, and salicylic acid did not effect PGA binding.

2. METHF

The binding of C-14 labelled METHF to normal human sera has been reported by Spector et al., (1975) Serum binding was determined by ultrafiltration at 23°C with a head pressure of 5 psi Nitrogen. Sixty to seventy percent of the drug was reported to be bound, and was constant over the concentration range tested. In addition, phenytoin, probenecid, and aspirin were found not to alter the binding of METHF.

3. LEU

The protein binding of leucovorin has not been reported.

VI. EXPERIMENTAL

I. Materials and Equipment

A. Drugs

Calcium Leucovorin, Lederle Laboratories, Pearl River, NY.

Methotrexate, Lederle Laboratories, Pearl River, NY.

dl-N-5-methyltetrahydrofolic acid, Barium salt, Sigma Chemical Co., St. Louis, MO.

l-Ascorbic acid, Sigma Chemical Co., St. Louis, MO.

Folic Acid, Sigma Chemical Co., St. Louis, MO. Gamma-amino-butyric acid, Sigma Chemical Co., St. Louis, MO.

Dihydrofolic Acid, Sigma Chemical Co., St. Louis, MO.

Tetrahydrofolic Acid, Sigma Chemical Co., St. Louis, MO.

Methylene Tetrahydrofolic acid was obtained from the National Institutes of Health, Bethesda Md.

B. Reagents

Sorenson's Phosphate Buffer, pH 7.4, was prepared by mixing together 1/15 M monopotassium phosphate (576 ml.) and 1/15 M disodium phosphate (424 ml.)

Paired-ion reagents, 1-pentane, 1-heptane, and 1-octane sulfonic acid, Pic B5, B7, and P8 respectively were obtained from Waters Associates, Milford, MA.

C. Chemicals

Ammonium Phosphate, dibasic, A.C.S, Fisher Scientific Co., Fairlawn, NJ.

Methanol, UV Grade, Waters Assoc., Inc., Milford, MA.

Acetonitrile, UV Grade, Waters Assoc., Inc., Milford, MA.

Disodium ethylenediamine tetraacetate, A.C.S., Allied Chemical, Specialty Chemicals Division, Morristown, NJ.

Phosphoric Acid, 85, A.C.S, Fisher Scientific Co., Fairlawn, NJ.

Potassium Hydroxide, A.C.S., Fisher Scientific Co., Fairlawn, NJ.

Sodium Phosphate, Dibasic, A.C.S., Fisher Scientific Co., Fairlawn, NJ.

Potassium Phosphate, Monobasic, A.C.S, Fisher Scientific Co., Fairlawn, NJ.

Tetrabutylammonium Chloride, Eastman Kodak Co., Rochester, NY.

Oxalic Acid, A.C.S, Fisher Scientific Co., Fairlawn, NJ.

Glacial Acetic Acid, A.C.S, Fisher Scientific Co., Fairlawn, NJ.
Nitric Acid, A.C.S, Fisher Scientific Co., Fairlawn, NJ.

Crystalline Human Serum Albumin, Calbiochem, Inc., San Diego, CA.

Crystalline Bovine Serum Albumin, Calbiochem, Inc.,
San Diego, CA.

Spectroscopic Powder, grade SP 2, Union Carbide Corporation, New York, NY.

Ceresin Flakes, Matheson Chemical Company, Lyndhurst, NJ.

D.   Equipment

Ultrafiltration Apparatus, Model 8MC, Amicon
Corp., Lexington, MA.

Ultrafiltration Membranes, 10,000 mw cutoff,
PM-10, Amicon, Corp., Lexington, MA.

Dialyzer Tubing, 2.5 cm. wide, 1.6 cm. diameter,
Fisher Scientific Co., Fairlawn, NJ.

High Pressure Liquid Chromatographic Pump, Model
M-6000A, Waters Assoc., Inc., Milford, MA.

uBondapak C18 reverse phase stainless steel
column, Waters Assoc., Inc., Milford, MA.

Radial Pak A Liquid Chromatographic Column, 5 mm.
I.D., Waters Assoc., Inc., Milford, MA.

Radial Compression Module, Waters Assoc., Inc.,
Milford, MA.

Sep-Pak, C-18 Cartridges, Waters Assoc., Inc.,
Milford, MA.

HPLC Loop Injector, Model 7120, Rheodyne, Inc.,
Berkley, CA.

Constant Temperature Drying Oven, Thelco Model 15,
Precision Scientific Co., Chicago, IL.

Digital Multimeter, B and K Precision Model 2800,
Dynascan, Corp., Chicago, IL.

Active Noise Filter, Model Sc102, Foxboro
Analytical Division, North Haven, CT.

Series-Parallel RC Combination Box, Eico, model
1140, Electronic Instruments Co., Long Island
City, NY.

Electronic Controller Model LC-2a, Bioanalytical
Systems Inc., W. Lafayette, IN.

Plexiglass Paste Cell, Model Tl-3, Bioanalytical
Systems Inc., W. Lafayette, IN.

Kel-F, Glassy Carbon Cell, Model Tl-5A,
Bioanalytical Systems Inc., W. Lafayette, IN.

Silver-Silver chloride Reference Electrode, Model

RC-1, Bioanalytical Systems Inc., W. Lafayette, IN.

Sample Concentrator Model 190, Fisher Scientific Co., Fairlawn, NJ.
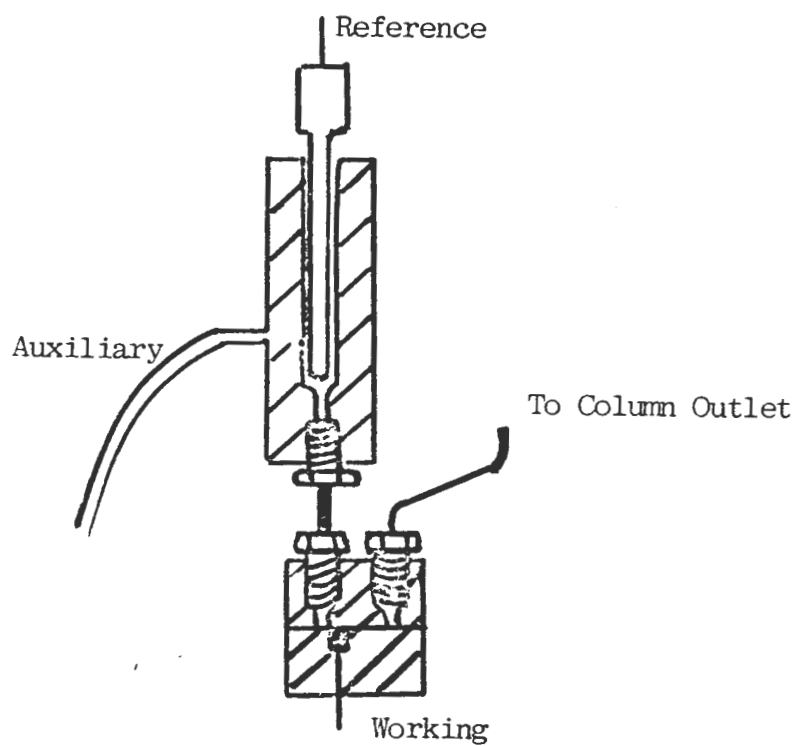
Figure 5: Diagram of the Electrochemical Flow-Through Cell

and auxiliary electrodes were downstream from the working electrode in this arrangement. The completed unit was mounted inside an aluminum box (20 cm. x 15 cm. x 11.5 cm.) which served as a support mechanism for the cell.

b. Electronic Connection

The electrochemical cell was connected to the LC-2A controller in the following manner. The four leads extending from the controller unit were fitted with banana plugs, one each for the working, reference, and auxiliary electrodes, and the fourth for ground. Four corresponding banana jacks were installed on one side of the support box. One jack served as a ground, to each of the remaining three a short piece of insulated wire was soldered and connected to the individual electrodes using miniature alligator clips. Each lead was tested to insure continuity of current flow before use.

The LC-2A output jacks were connected to an ANALABS, Model SC 102 active noise filter, which served to reduce the residual current (background noise) inherent in the detector. The output from the noise filter was coupled to the appropriate input terminal of the electronic integrator.

A 0.8 volt potential was applied to the working electrode for routine quantification of LEU and METHF. For most applications the summing amplifier was set at 50 nanoamps/volt although in some cases the 20 nanoamp/volt

scale was used. The active noise filter remained set at a constant gain of ten which boosted the input signal to the integrator approximately tenfold.

Oxidation of the mobile phase components, i.e, supporting electrolyte, trace metals, and dissolved oxygen produced a background current ranging from 30 to 70 nanoamps/volt.

c. Electrode Preparation

i. Carbon Paste Electrodes

The method of preparing carbon paste electrodes was identical whether graphite/mineral oil, silicone oil, or wax-impregnated graphite/oil pastes were used. The working electrode block was carefully cleaned with water and methanol, taking care to remove any old paste from the electrode well. The cell was then dried carefully with Kimwipes and further allowed to air dry. A few milligram sample of paste material was placed on a folded computer card or piece of glassine paper. The paste was then poured into the three mm. diameter electrode well in several portions. A wooden applicator stick, two to three mm. in diameter, was used to tamp down the paste between additions. This process was continued until the paste was approximately level with the cell surface. A small pile of paste was then placed on a clean computer card and the inverted electrode block pressed onto the pile. Using random motion the block was moved around the card until a

flat, smooth surface was obtained. The entire electrochemical cell was then reassembled as described. Special care was given to keep electrode paste away from other cell surfaces and to avoid scratching the cell surface.

After connecting all electrodes and the ground, the appropriate voltage was impressed between the working and reference electrodes. The rate at which the cell surface was "conditioned", i.e., the time required for background current to decay to the 20 to 70 nanoamp/volt range varied. In many cases one and one-half to three hours was required, in some instances the residual current did not decrease to acceptable levels after overnight operation, in which case the electrode was repacked.

ii. Glassy Carbon Electrode

Regeneration of the highly polished surface of a glassy carbon electrode was accomplished in any one of several ways. After long periods of continuous operation, i.e., six to eight weeks, a decline in detector sensitivity was often noted. Examination of the electrode surface showed a dulling of the mirror finish which has been attributed to the formation of oxide films and adsorption of products and reactants (Fleet and Little, 1975). In most cases simply rinsing the cell surface with distilled water, followed by methanol was sufficient to restore the cell surface.

In some cases the treatment detailed above did not restore the cell surface and polishing was required. The glassy carbon was first washed with distilled water and left wet. A small quantity, one to two ml. of jeweler's alumina paste was placed on a felt pad and wetted slightly. The glassy carbon surface was then impressed upon the paste and the entire electrode block moved in a random motion about the pad for approximately one minute. The carbon surface was then rinsed with distilled water and a drop of 6N Nitric Acid was placed on the carbon and allowed to stand for 30 minutes. The cell was then rinsed with water and methanol, allowed to air dry, and reassembled.

A potential was then applied to the working electrode for a minimum of four hours before quantitative determinations were attempted; in most cases the detector required overnight operation to become "conditioned".

If polishing the carbon electrode with alumina did not regenerate the cell surface, an additional step was added to the polishing procedure. A small amount of a diamond dust slurry was placed on a disc of "660 wet dry" abrasive. The cell surface was "rough" polished by this method before progressing to the alumina paste and subsequent steps as described above.

Once a potential was impressed between working and reference electrodes, the system remained charged until the electrode surface required regeneration.

2. Chromatography

a. Apparatus

The liquid chromatograph consisted of: a Waters Associates Model 6000A solvent delivery system; a Rheodyne Model 7120 syringe loading sample injector equipped with a 100 ul loop; either a Waters Assoc. uBONDAPAK C-18 reverse phase column (4mm x 30cm), or an 8mm I.D. RADIAL-PAK C-18 cartridge (13mm x 10cm) and RCM-100 Radial Compression Module; Thelco Model 15, constant temperature oven; and a BAS Model LC-2A, electrochemical detector. The 1 volt output of this detector was "stepped-down" and connected to a Waters Assoc., "Data Module", electronic integrator.

b. Solvent Systems

Paired-ion chromatography was used for the separation and quantification of LEU and METHF in aqueous systems and from serum. Two classes of pairing agents; pentane, heptane, or octane sulfonic acid, and dibasic ammonium phosphate $(NH4)2HPO4$ were evaluated for their suitability in the determination of reduced folates.

Sulfonic acid buffers were prepared by pouring the contents of one vial of pre-mixed paired-ion reagent, PIC B5, B7, B8 (Waters Assoc.) into a liter volumetric flask which was then brought to volume with distilled-deionized water, to yield a final paired-ion concentration of 5mM. The pH of this buffer system was maintained at 3.5 and was

used without further modification. The organic component of mobile phases which contained sulfonic acids ranged from 10% to 40% methanol and 0% to 10% acetonitrile. The final mobile phase was prepared by adding an appropriate volume of organic solvent (CH3OH or CH3CN) to a 1 liter volumetric flask which was then brought to volume with the previously prepared sulfonic acid reagent solution.

Dibasic ammonium phosphate, (NH4)2HPO4, was also used as a "paired-ion" reagent. Several concentrations of ammonium phosphate between 0.005M and 0.5M were evaluated. An appropriate amount of reagent was placed in a volumetric flask and brought to volume using distilled-deionized water. prepared as described above. The pH of ammonium phosphate mobile phases was varied between pH 3.5 and pH 7.0, and was adjusted after the mobile phase; buffer solution plus organic components, had been prepared. No corrections were made for the different amounts of H3PO4 which were added to bring the mobile phase to the desired pH.

Sodium EDTA has been reported to be valuable in quieting "electrochemical detector noise", (R. Shoop, 1978) presumably by chelating trace metals which may be found in the mobile phase components or in the chromatograph itself. Ten mM Sodium EDTA was therefore added to each mobile phase as a "precautionary measure".

All mobile phases were freshly prepared on the day they were to be evaluated. Following preparation each

mobile phase was filtered through a Millipore Type-FH, 43mm filter, which had been wetted with methanol. The mobile phase was stored in glass Erlenmeyer flasks and covered with Parafilm during use.

3. Protein Binding

a. Preparation of the Membrane

The ultrafiltration membrane was soaked in distilled, deionized water for one hour to remove the glycerin coating the membrane. The membrane was then placed in the ultrafiltration apparatus, seven ml. of water (one cell volume) was forced through the membrane, followed by an equal volume of Sorenson's buffer, pH 7.4. The interior of the cell, membrane surface, and teflon tubing through which the filtrate emerges were dried with a Kimwipe before beginning a binding determination.

b. Sample Preparation

Human serum albumin was prepared as an eight percent (w/w) solution with Sorenson's buffer, pH 7.4, twenty four hours prior to a protein binding determination. The protein was wetted with buffer and gently agitated to aid dissolution. Foaming of the solution was avoided since this can result in surface denaturation of the protein. Fifty ml. of the albumin solution was then poured into a 25 cm. section of dialysis membrane which had been previously soaked in buffer solution and knotted at one end. The dialysis tube was tied off and placed in a one liter flask

filled with Sorenson's buffer, pH 7.4, tightly covered and stored at40c until used.

Bovine serum albumin was prepared in Sorenson's buffer, pH 7.4, as a four percent (w/w) concentration, the normal concentration of albumin in serum. The binding of leucovorin to bovine albumin was carried out using the ultrafiltration technique described previously. The range of total drug concentrations studied was from $2.5x10^{-4}$ M to $1.0x10^{-4}$ M. Samples were assayed in duplicate immediately after ultrafiltration.

Leucovorin, 5-methyl THF and methotrexate solutions were prepared daily using Sorenson's buffer, pH 7.4. Drug stock solutions were stored in tightly capped, opaque vials, to prevent light catalyzed oxidation and were stored on ice during the binding experiment.

c. Ultrafiltration

Equal volumes (three and one-half ml.) of drug and albumin solutions were pipetted into the previously treated ultrafiltration cell. The final albumin concentration produced in this manner was four percent (w/w). The drug-protein solution was stirred for approximately 30 to 45 seconds before beginning the ultrafiltration and then throughout the entire filtration process. Stirring insures complete mixing of the drug and albumin solutions before beginning filtration and prevents "concentration polarization", i.e., accumulation of the retained protein

at the membrane surface creating a "boundary layer". The accumulated protein restricts the flow of solution through the membrane, alters the filtration characteristics of the system and slows the rate of filtration (Anonymous, Amicon Product Literature, 1980).

Nitrogen gas (20 psi) was applied to the cell, forcing the solution across the membrane. The first four drops of filtrate, approximately 200 microliters, was discarded, the following 400 to 500 microliters was collected in a 12 x 75 mm. glass test tube, covered, protected from light and stored on ice until analyzed. All samples were analyzed on the day the ultrafiltration was carried out. The ultrafiltration cell was washed with Sorenson's buffer, pH 7.4, between each binding determination and a small quantity of buffer was forced through the membrane. The cell was then dried as previously described in preparation for the next analysis. Experiments were always carried out in order of increasing total drug concentration to minimize potential error due to drug carry-over from the previous sample. Each binding experiment was carried out in triplicate. Samples were assayed twice and the average value used to determine the free drug concentration. In order to examine non-specific binding, i.e., the binding of drug to material other than albumin, notably the ultrafiltration cell or membrane, seven ml. aliquots of leucovorin and 5-methyl THF, in Sorenson's buffer, pH 7.4, were pipetted into the cell and filtered as previously

described. Quantitative analysis was performed on the pre and post filtered samples to detect any changes in drug concentration following filtration.

Three and one-half ml. each of albumin solution and Sorenson's buffer, pH 7.4, (drug free) were pipetted into the ultrafiltration cell and filtered. The collected filtrate was analyzed to detect any components eluting from the albumin which might interfere with the quantification of leucovorin or 5-methyl THF.

4. Human Studies

a. Human Subject Protection

A detailed report of the proposed project, its objectives, methods and potential hazards was submitted to Human Studies Committees and Institutional Review Boards at both the University of Rhode Island and Roger Williams General Hospital (Appendix I). Each subject who volunteered for the study was provided with a copy of the experimental protocol and was required to read and sign an informed consent form which explained the study in detail (Appendix II).

b. Subject Selection

Six subjects meeting the criteria described in the experimental protocol were selected for inclusion in the study. All subjects, three males and three females, were healthy volunteers between the ages of 28 and 33. They

weighed between 45 Kg and 100 Kg. and body surface areas were between 1.37 m² and 2.28 m². The volunteers were free from known hypersensitivity to folic acid or its derivatives and had no medical abnormalities which would, in the opinion of the attending physician, add further risk to the subjects or complicate the study.

Participants were required to abstain from all drugs, including alcohol, for 48 hours prior to the test period. In addition, the participants were asked to fast for 12 hours before drug administration and two hours after dosing.

Leucovorin was prepared for intravenous injection by reconstituting 50 mg. of Calcium Leucovorin (Lederle Laboratories, lot no. 616-302) with 5 ml. of sterile water for injection to yield a final concentration of 10 mg. ml.$^{-1}$ Each subject received 10 mg. of drug immediately after a "zero-time" or "blank" blood sample was obtained. The collection of subsequent blood samples was scheduled as follows; 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes. The actual time of each sample collection was documented so that actual sampling times were available for subsequent analyses.

c. Cancer Patients

Patients undergoing high dose methotrexate-leucovorin rescue therapy under the experimental protocols established by the Oncology Section at Roger Williams General Hospital,

(Appendix III) were evaluated for possible inclusion in the project.   Nine patients were admitted to the MTX program between November 1979 and November 1980; five of them were asked to participate in the LEU pharmacokinetics project. Table II details the diagnosis, age, sex, weight, and LEU dose received by each patient. Since these patients were already involved in an experimental protocol (e.g. high dose MTX), participation the the current project required only the patient's consent to obtain appropriate blood samples following the administration of LEU. This was considered by the physicians involved to constitute no additional risk to the patients. All patients were treated in the clinical oncology unit of the hospital where their blood samples were obtained by the regular nursing and support staff personnel in the same manner as for the "normal" volunteers.   All patients were free to terminate the blood sampling at any time, for any reason.

d. Method of Blood Sample Collection

An intermittent infusion set, 19G x 2.22 cm., was inserted by a physician into a vein on the subject's forearm and carefully taped in place. After drawing a zero time, or blank sample, the catheter was filled with 0.5 mL of a dilute, 100 units ml^{-1}, heparin solution in order to prevent blood clot formation within the tube. At the time of sample collection a latex tourniquet was tightened on the subject's arm above the position of the catheter, and

TABLE II

METHOTREXATE PATIENT INFORMATION

Patient	Age	Sex	Diagnosis	Methotrexate Dose
F.F.	48	M	Osteogenic Sarcoma	3.71 mg IV
L.M.	56	M	Osteogenic Sarcoma	4.30 mg IV
P.F.	26	F	Choriocarcinoma	1.78 mg IV
F.Y.	62	M	Osteogenic Sarcoma	12 mg I.M.

the heparin solution was withdrawn from the catheter with a 1 ml. disposable syringe. Using a fresh 5 ml. disposable syringe with a 21G needle, a 3 to 5 ml. blood sample was carefully withdrawn and placed in a 10 ml. "yellow-top" vacutainer tube containing 5 mg of ascorbic acid. The catheter was immediately flushed with the dilute heparin solution which remained in the catheter until the time of the next sample collection.

Blood samples were allowed to clot for at least 1 hour at room temperature, and were then centrifuged for 10 minutes at 2000 rpm. The serum was carefully removed and placed in 12mm. x 72mm. disposable glass test tubes to which had been added 20 ul. of a 100 mg. ml^{-1} solution of ascorbic acid. The test tubes were then covered with Parafilm and stored frozen until assayed.

e. Extraction Procedure

Leucovorin was extracted from plasma or serum samples in the following manner. A Waters C-18 Sep-Pak cartridge was connected to a 10 ml. disposable syringe from which the plunger had been removed. Ten ml. of methanol was added to the syringe and, using the plunger, slowly pushed through the Sep-Pak. When all the methanol had eluted from the Sep-Pak, the cartridge was gently removed from the syringe and the plunger withdrawn once again. After reconnecting the Sep-Pak cartridge, the process was repeated using 10 ml. of a 0.005M (NH4)2HPO4 buffer, pH 5.5. Again, care was

taken not to force the fluid completely out of the cartridge before removing it from the syringe.

Following the above two step conditioning process, the plasma or serum sample was loaded through the barrel onto the Sep-Pak, to be followed by 1 ml. of the ammonium phosphate buffer. This mixture was then slowly forced through the cartridge and the effluent was discarded. An additional 3 ml. of buffer was then eluted through the Sep-Pak. However, in this case the plunger was pushed through the barrel with sufficient force to elute essentially all solution from the Sep-Pak. The effluent from this step was also discarded. The outlet end of the cartridge was then connected to a vacuum tube and the Sep-Pak was air-dried for 5 minutes.

The dried Sep-Pak was then eluted with 2 ml. of methanol and the eluent collected in sample dishes designed for use in a Fisher Model 190 sample evaporator. The sample dishes were placed in the evaporator which had been previously heated to 50°C and then placed under a vacuum. Nitrogen gas was then allowed to flow slowly into the evaporator until all samples had been evaporated to dryness. An additional 200 ul. of methanol was added to each dish; any residue from the sides of the dish was washed toward the bottom. This volume of methanol was also evaporated to dryness. Each sample was then covered with Parafilm, placed in a dessicator, and stored frozen, and protected from light until assayed.

VII. RESULTS and DISCUSSION

The electroactivity of a wide variety of organic functional groups i.e., hydrocarbons, amines or amides, phenols, catecholamines for oxidation, and esters, ketones, aldehydes, diazo, and nitro compounds for reduction have been reported (Fleet and Little, 1977). However, the electrochemistry of complex organic molecules is not well understood (Kissinger, 1977). The suitability of electrochemical detection for a compound is best determined by a voltammogram in which current (generated by the oxidation or reduction of an electroactive species) is plotted as a function of applied voltage.

Samples of calcium leucovorin and methotrexate were shipped to Bioanalytical Systems Inc., W. Lafayette, IN., to determine the electroactivity of the folate analogs. Figures 6 and 7 are the resultant voltammograms. Calcium leucovorin was dissolved in 0.1 M acetate buffer and subjected to an oxidizing potential. No current was detected until the applied potential reached 0.5 volts. The current maximum, approximately 20 nanoamps, was achieved at 0.6 volts. Both figures show an apparent decline in current at higher potential. This is the result of the depletion of oxidizable species in the sample; any applied potential greater than 0.6 volts would be expected to generate the same current.

Methotrexate, dissolved in 1 M acetate buffer (with
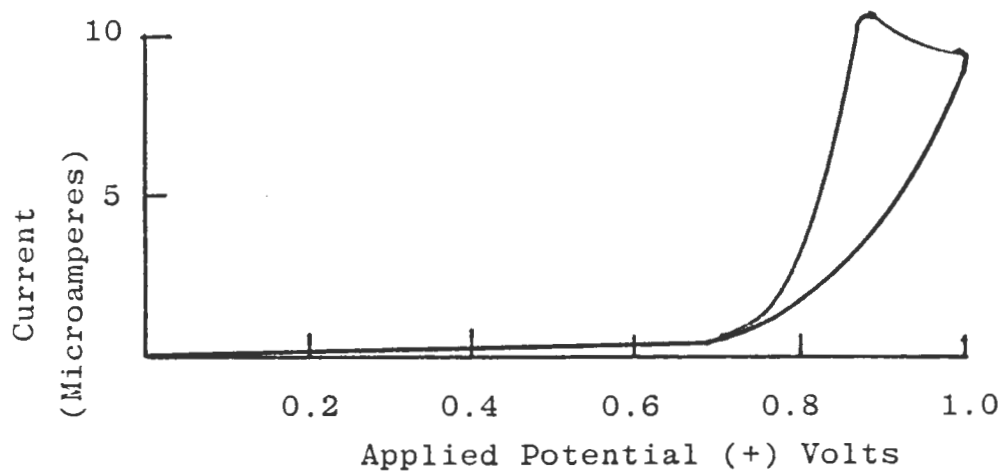
Figure 6:   Leucovorin Hydrodynamic
           Voltammogram
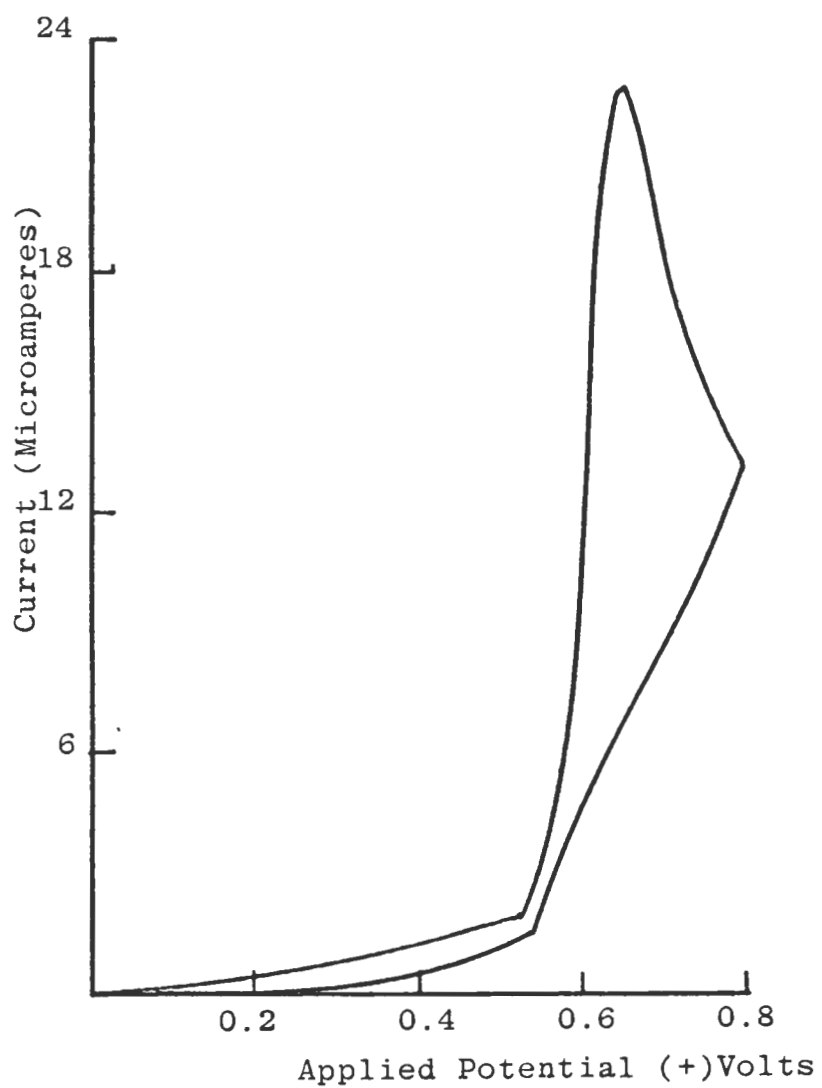
Figure 7: Leucovorin Hydrodynamic
Voltammogram

twenty percent ammonium carbonate (0.1M) to aid dissolution) produced a peak current of approximately 10 microamps at a potential of 0.8 volts. As was the case with LEU, the current declined rapidly due to depletion of electroactive species.

Although both compounds were examined under the same conditions, e.g., voltage scan rate, working electrode, and reference electrode, different media were used to dissolve the drugs. Since the bulk solution could vary in characteristics such as resistance and diffusion coefficients, it was difficult to reach conclusions concerning the relative electroactivities of leucovorin and methotrexate. However, the voltammograms confirm the electroactivity of the folate analogs and the feasability of using amperometry as a method of detection.

1. Electrode Evaluation

a. Carbon Paste, Silicone-Oil

Carbon paste, silicon oil base electrode material, Cp-O, was evaluated using mobile phases of 20% to 30% methanol in 5mM 1-pentane, 1-heptane, or 1-octane sulfonic acid, pH 3.5. The flow rates used ranged between 0.7 and 1.0 ml. per minute. At an applied potential of 1.0 volt, it was possible to measure routinely LEU concentrations of 5×10^{-7} M (50 nanograms). The apparent lower limit of sensitivity was 5×10^{-8} M.

The average useful life of a Cp-O working electrode

was only two to three days, contrary to the weeks or months of stability reported for this electrode (Kissinger, 1977). The working electrode was considered unusable when the background, or residual current was greater than 90 to 100 nanoamps per volt applied across the working electrode. Because four to eight hours of continuous operation were frequently required for a newly packed or resurfaced electrode to stabilize to low residual currents, the Cp-O paste was considered to be unacceptable for routine applications.

The instability of the electrode surface has been attributed to an uneven surface in which paste can project into the mobile phase flow and flake apart, creating a high background noise. (Keller, et al., 1976). Furthermore, during the packing process, it is possible for some paste to become imbedded in surface imperfections on the electrode block, creating additional residual current (Keller et al., 1976). Air bubbles may become trapped within the paste material during the cell packing process, these bubbles may also contribute to high background current and decreased useful life (Kissinger, 1977). Electrode to electrode reproducibility was inadequate, a result of the manner in which the electrode well was packed and the surface prepared. Using the method of cell preparation described previously, electrodes were produced with lower limits of sensitivity ranging from 5.0×10^{-8} M to 1.0×10^{-5} M. The Cp-O paste was considered unsuited for

routine use under the chromatographic conditions employed because of the short useful life and the inability to produce electrodes with consistent levels of sensitivity.

       b. Wax-Impregnated Graphite with Silicone Oil

One investigator claims that wax-impregnanted graphite/silicone oil (Cp-W) electrodes are more durable and more easily resurfaced than previously used carbon pastes (Mosher, 1978). A supply of wax-impregnated paste, prepared by the method of Atuma and Lindquist (1973) was obtained from Pfizer Laboratories, the generous gift of Frank Mosher.

The wax was evaluated, using mobile phases containing 20% to 30% methanol in 5 mM sulfonic acid reagents, pH 3.5. Mobile phase flow rates ranged from 0.7 to 1.0 ml. per minute. At an applied potential of 1 volt, the lower limit of detection which was achieved with the wax, was comparable to the Cp-O pastes. The baseline noise, or residual current, produced with this electrode was lower than that with the previous system; fewer noise spikes or random peaks were generally observed.

The relatively low residual current may be attributable to the ceresin content of the electrode material which results in a stiffer paste than do mineral or silicone oil-based electrodes. The harder paste can be rigorously "polished" and clearly provides a more uniform and smooth surface than other pastes. Furthermore, because

it is a stiffer material, the wax electrode material is apparently not as susceptible to distortion caused by the motion of mobile phase flowing over its surface. Such distortion is one of the major causes of excessive residual current (Kissinger, 1977).

The average useful life of wax-impregnanted pastes was significantly longer than for oil-based electrodes. Two to three weeks of continous operation were frequently possible with Cp-W electrodes. As with Cp-O electrodes, when background currents exceeded 90 to 100 nanoamperes per volt, it became necessary to resurface or completely repack the electrode well. Several hours were required to establish a stable baseline current in the range of 20 to 30 nanoamperes per volt following the packing of a new electrode. Increasing the appplied potential to the maximum 1.2 volts did not significantly affect the time required for an electrode to be adequately conditioned. It was not possible to pack the electrode cell and achieve consistent, reproducible detector responses. Two or three cell packings were often necessary to produce an electrode with a suitable sensitivity and residual current. Despite this disadvantage, Cp-W was routinely used under the chromatographic conditions described above, since an adequately conditioned cell had a useful life five to ten times that of Cp-O electrodes.

In order to improve the resolution between LEU and METHF it became necessary to add acetonitrile to the mobile

phase. Cp-W electrode paste is not stable in mobile phases containing large amounts of organic solvents notably methanol or acetonitrile. Exposure to the ternary mobile phase resulted in the rapid destruction of the electrode surface rendering Cp-W inadequate for further use in detecting the reduced folates.

The continued use of wax-impregnanted paste was further hampered by the inabiliy to produce a suitable material using the reported method (Atuma and Lindquist, 1973). Although several batches of the wax-impregnated paste were manufactured, each resulted in a residual current greater than 100 nanoamperes per volt which could not be offset electronically, thus rendering the electrode useless.

c. Glassy Carbon

The lack of reproducibility among electrode surfaces and short useful life inherent in paste electrodes prompted a search for alternate electrode material. Fleet and Little (1977) suggested that solid electrode voltammetry may be a useful alternative to pastes and reported a cell design using a piece of highly polished (glassy) carbon as a working electrode. Lankelma and Poppe (1976) and Hashimoto and Maruyama 1978 have reported on the utility and stability of glassy-carbon electrodes.

i. Electroactivity of Folate Analogs

A five mm. glassy-carbon working electrode was obtained from Bio-Analytical Systems Inc. and evaluated with mobile phases containing four to forty percent methanol, three to fifteen percent acetonitrile and having pH values ranging from 3.5 to 7.0. The applied potential varied from 0.1 to 1.0 volts. The electrode was exposed to mobile phase flow rates as high as 8.0 ml. per minute without apparent damage to the cell surface.

The electroactivity of LEU and METHF was determined over an applied potential range of 0.1 to 0.9 volts. The summing amplifier was set an 50 nanoamperes per volt. Chromatographic conditions were; mobile phase II, column temperature 37°C, and a flow rate of 3.0 ml. per minute.

Figure 8 is a plot of detector response, measured as log peak height per mole of drug, as a function of applied potential. No current was generated from LEU until the applied potential reached 0.4 volts. The current produced by LEU increased from 0.4 volts to an apparent plateau between 0.7 and 0.8 volts. The residual current resulting from an applied potential of 0.9 volts was greater than 100 nanoamperes per volt and therefore could not be compensated for electronically. As a result current produced by the oxidation of leucovorin could not be quantified above 0.8 volts.

METHF, unlike LEU, did not produce a measureable

current until the applied potential reached 0.3 volts. Between 0.3 and 0.8 volts the current produced by the oxidation of METHF remained constant. It is apparent from figure 8 that, under the electrochemical and chromatographic conditions described above, METHF is a more readily oxidized compound than is LEU. Further, if chromatographic methods were not able to resolve the two folates, METHF could be quantified by operating at an applied potential of 0.3 volts.

Under the chromatographic and electrochemical conditions described previously, and at an applied potential of 0.8 volts, it was possible to generate reproducible standard curves of peak height as a function of concentration for both LEU and METHF. A linear response was obtained for both drugs over the concentration range of $2.5 \times 10^{-7}M$ to $3 \times 10^{-6}M$. Correlation coefficients in both cases were routinely greater than 0.99. Analysis of variance of linear regression showed a significant correlation for both drugs ($p < 0.05$).

ii. Durability

The glassy-carbon electrode has an indefinite useful life. Routine operation with a five mm. diameter electrode has been carried out for sixteen months; no treatment other than renewal of the electrode surface has been necessary.

Auto-oxidation of the electrode surface and absorption of effluent material are the two factors most responsible
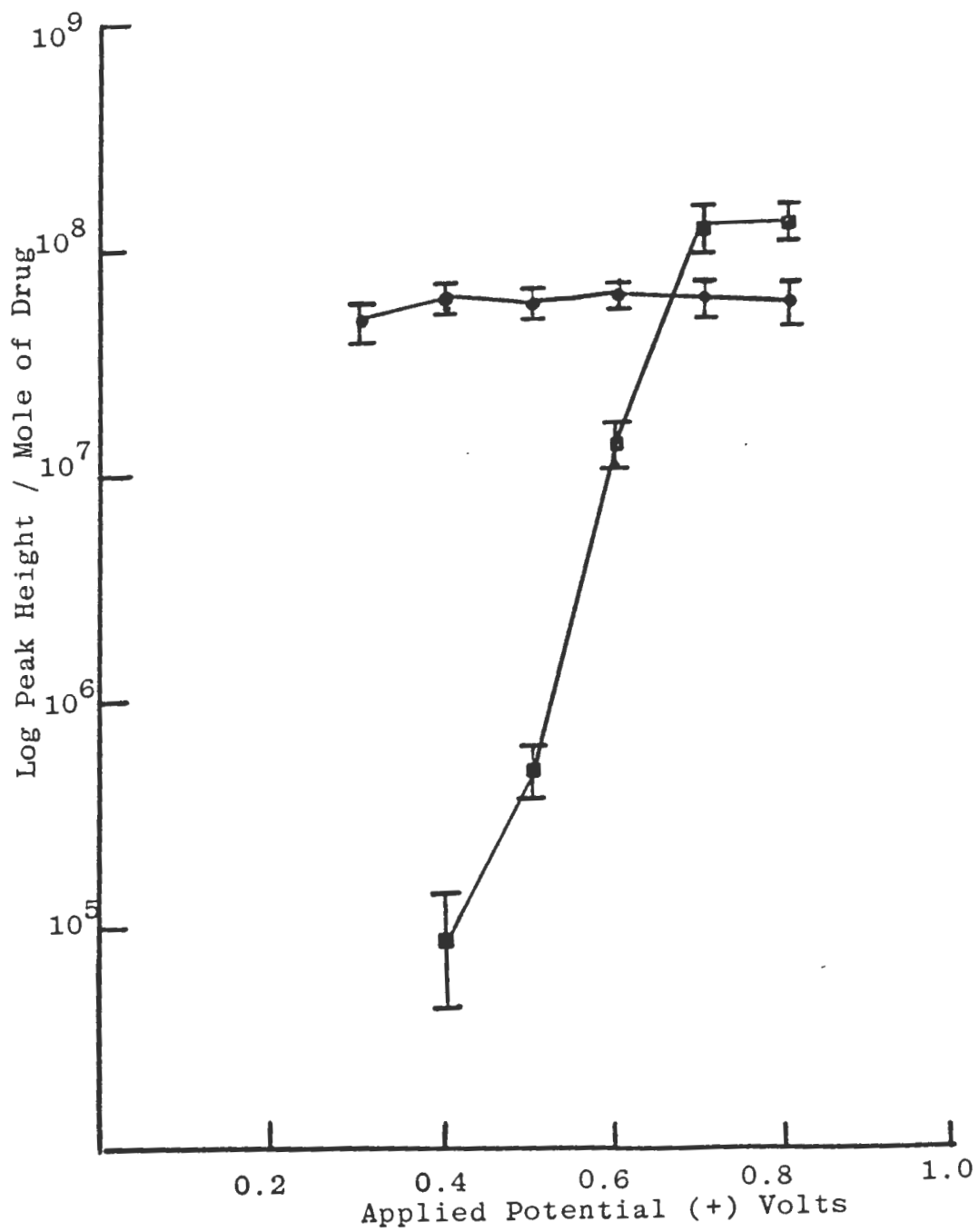
Figure 8: Peak Height as a Function of Applied Potential for Leucovorin and Methyl-tetrahydrofolate ●

for the decrease in sensitivity common to glassy-carbon electrodes (Fleet and Little, 1977). After several weeks of continuous operation a decline in residual current and sensitivity were observed. Figure 9 represents a series of standard curves, peak height as a function of concentration, generated from the regression coefficients for leucovorin. The constantly decreasing slope evident in A-D, figure 9 clearly demonstrates the loss in sensitivity over a six-day period of continous operation. Removal of the adsorbed material at the electrode surface is all that is usually necessary to reestablish the sensitivity of the electrochemical detector. This was accomplished by rinsing the cell surface with distilled water, followed by methanol, and allowing the cell to air dry before being reassembled. Cycling the applied potential between -2 and +2 volts has been suggested as a means of regenerating a glassy-carbon surface; however, this method was not successful. Mechanical removal of adsorbed material was the only adequate means of restoring the sensitivity of the system.

iii. Effect of Flow Rate

The effect of mobile phase velocity on the diffusion layer thickness (s) within the electrochemical cell and the resulting effect on current yield has been previously discussed. The optimal flow rate, in terms of detector response, for LEU and METHF was determined by plotting
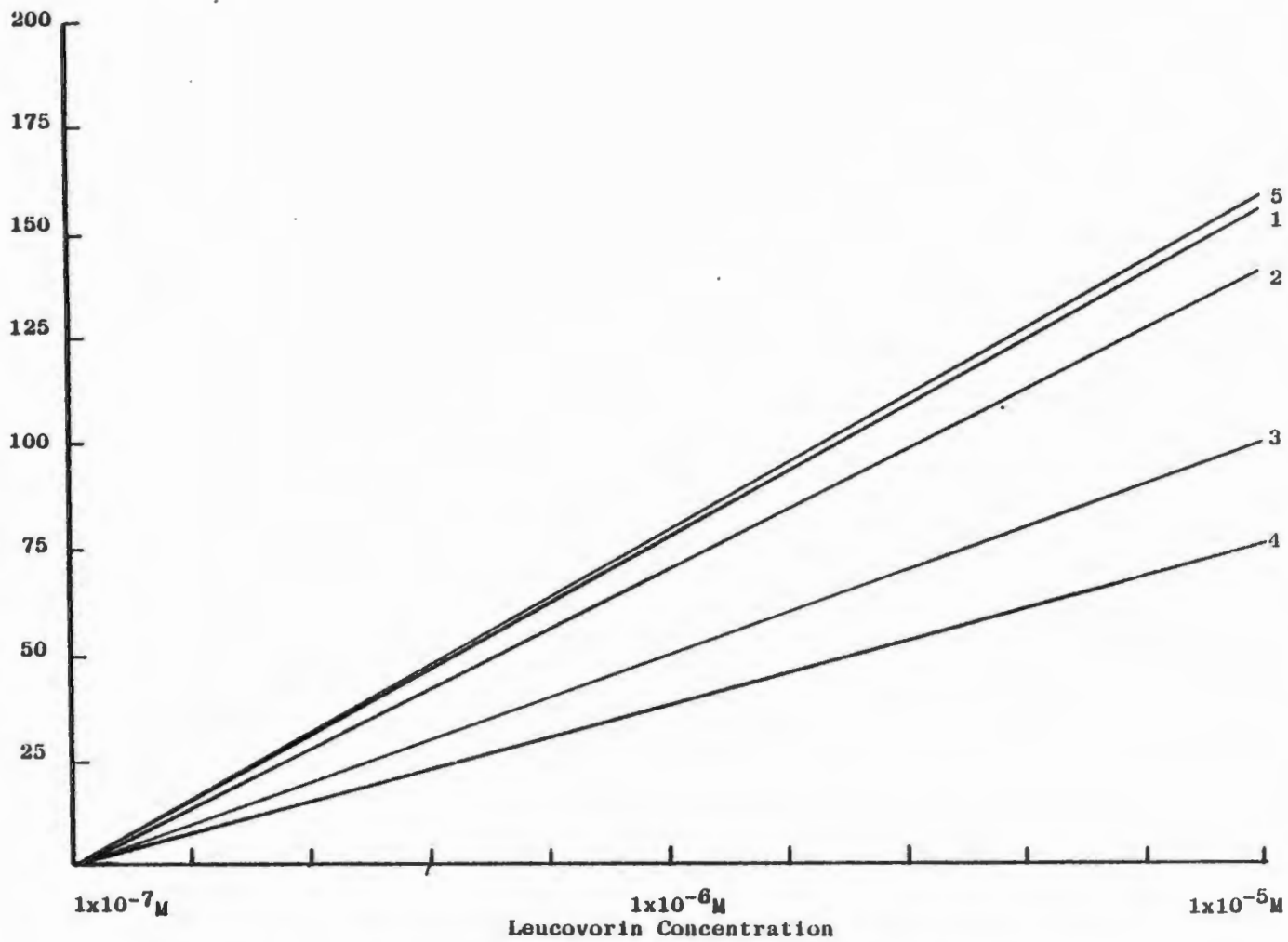
Figure 9; Decline in Detector Response Measured as Peak Height
as a Function of Time

response, measured as peak height, as a function of flow rate. It is apparent from Figures 10 and 11 (LEU and METHF respectively), that peak height decreases with increasing flow rate between 1.0 and 7.0 per minute. One way ANOVA showed that flow rate had a significant ($p < 0.05$) effect on peak height for both compounds. ANOVA with linear regression shows a significant linear effect ($p, 0.05$) for LEU (r^2 = 0.89, n=33) and for METHF (r^2 = 0.77, n=33). However, the analysis also showed a significant departure from regression ($p < 0.05$) for both drugs. This would suggest that the best model describing peak height as a function of flow rate should allow for curvilinear regression.

Although a flow rate of 1.0 ml per minute produced the optimal detector response for both LEU and METHF, 3.0 ml. per minute was chosen as the usual operating mobile phase velocity for two reasons. First, flow rates less than 3.0 ml. per minute resulted in unacceptably long chromatographic run times. Secondly, although statistically significant, the difference in peak height at 1.0 versus 3.0 ml. per minute was not considered to be of practical significance and, further, would not appreciably affect either the lower limit or reproducibility of the assay. The use of higher flow rates was precluded since rates greater than 3.0 ml. per minute resulted in loss of resolution between some early eluting peaks as well as loss in detector response evident in Figures 10 and 11.
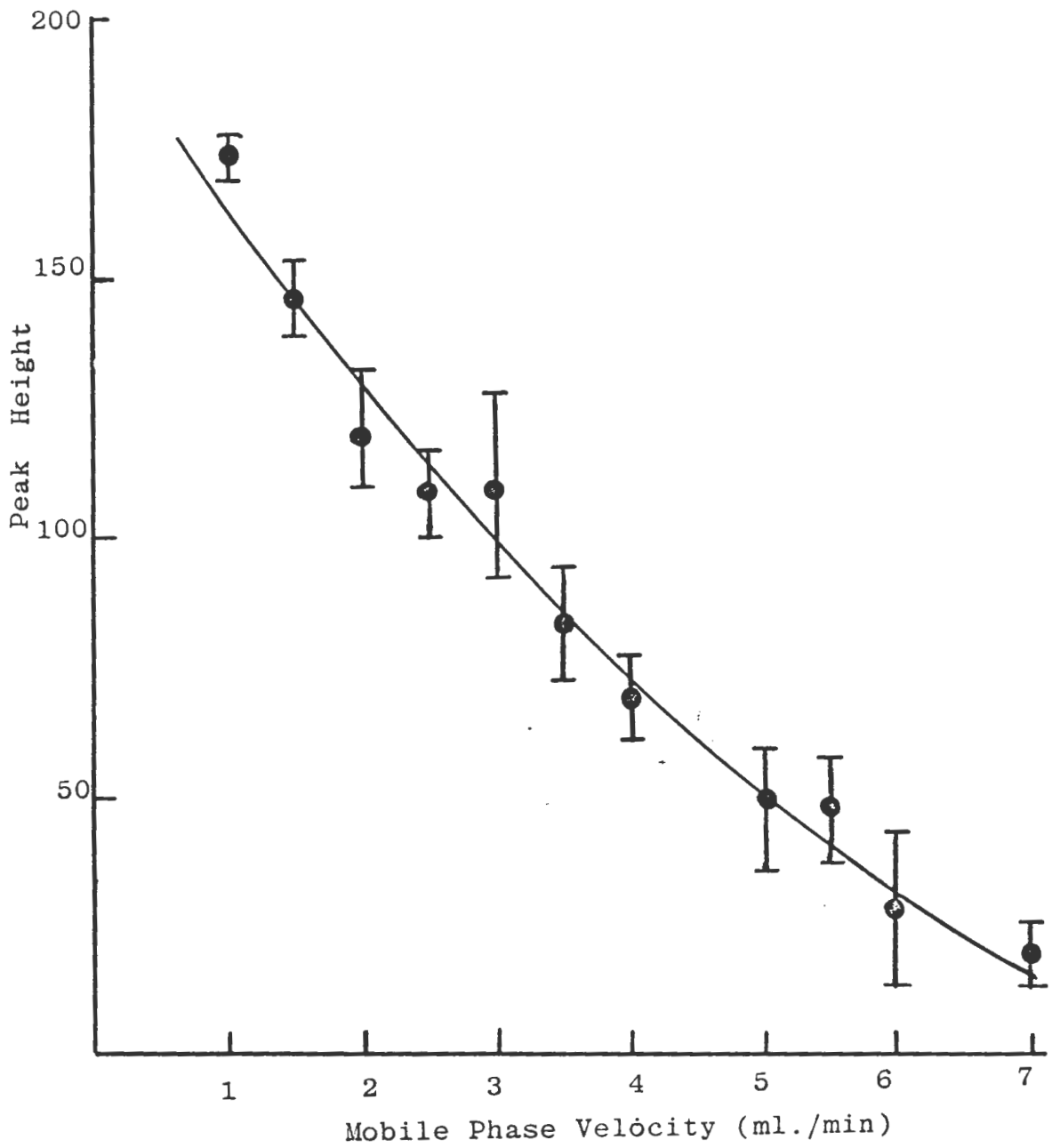
Figure 10: Effect of Mobile Phase Velocity on Detector Response to Leucovorin
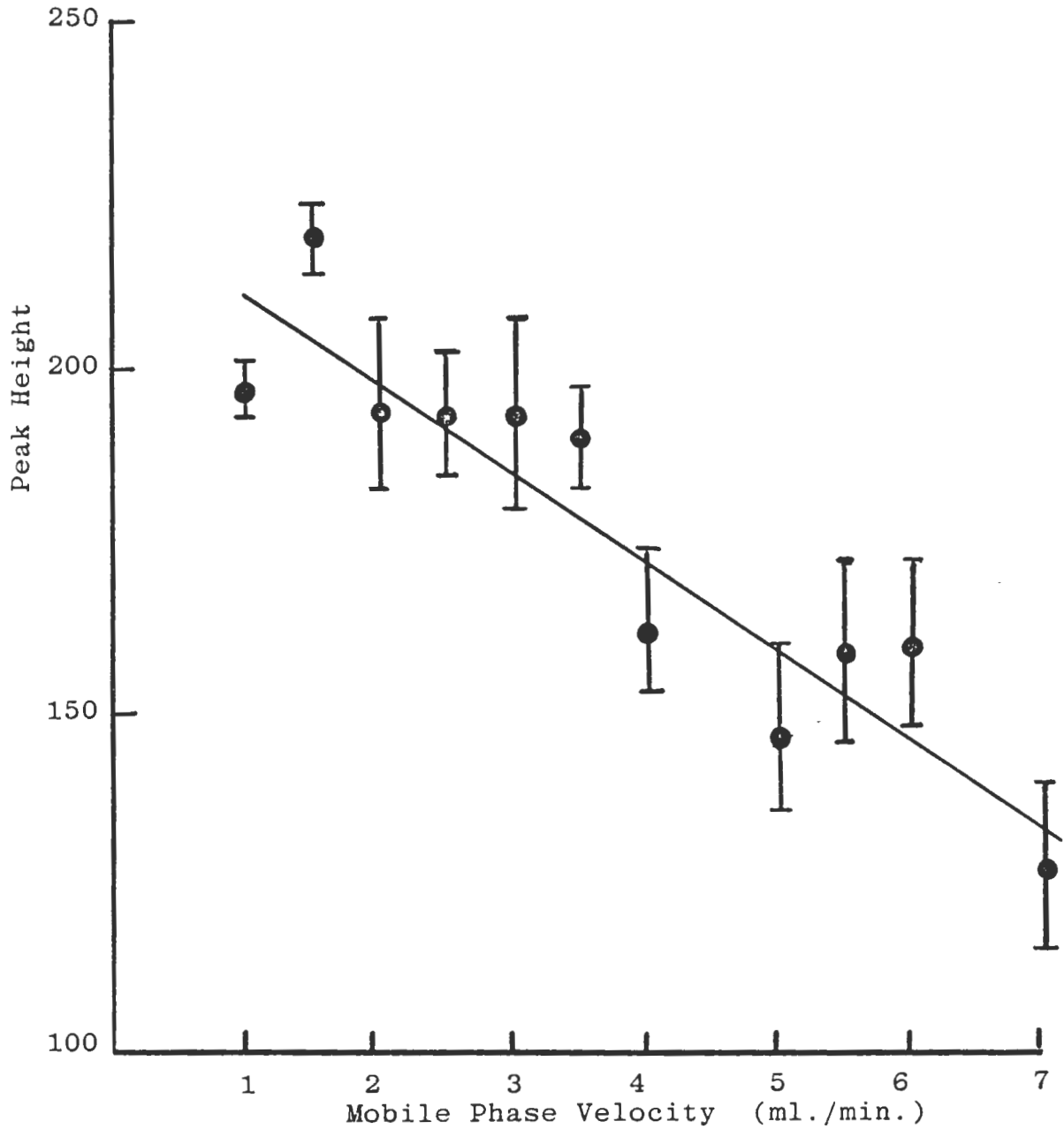
Figure 11: Effect of Mobile Phase Velocity on Detector Response to Methyltetra-hydrofolate

iv. Effect of Salt Concentration

High electrolyte concentrations are necessary in mobile phases to reduce resistance and produce the greatest current at the electrode. Varying concentrations of electrolyte may also be expected to affect the diffusion coefficient of the electroactive species, which according to equation 6, will affect the limiting current produced at the electrode.

Ammonium phosphate, dibasic, was the optimum paired-ion reagent in the separation of the reduced folates. The effect of varying salt concentrations on detector response was determined for LEU and METHF (Figures 12 and 13 respectively). The concentration of buffer ranged from 0.005 M to 0.5 M and was increased at one-half log intervals. The pH of the mobile phase was 5.5 and the flow rate was 3.0 ml. per minute.

One way ANOVA with linear regression showed a significant effect ($p < 0.05$) of mobile phase salt strength on response, measured as peak height for both LEU and METHF. The plot for leucovorin (Figure 12) is best described by curvilinear regression ($r^2 = 0.98$, n=15). There is a constant increase in response with salt concentration until an apparent plateau is reached between 0.1 M and 0.5 M salt concentration.

Figure 13 represents METHF peak height as a function of ammonium phosphate concentration. Rather than increase
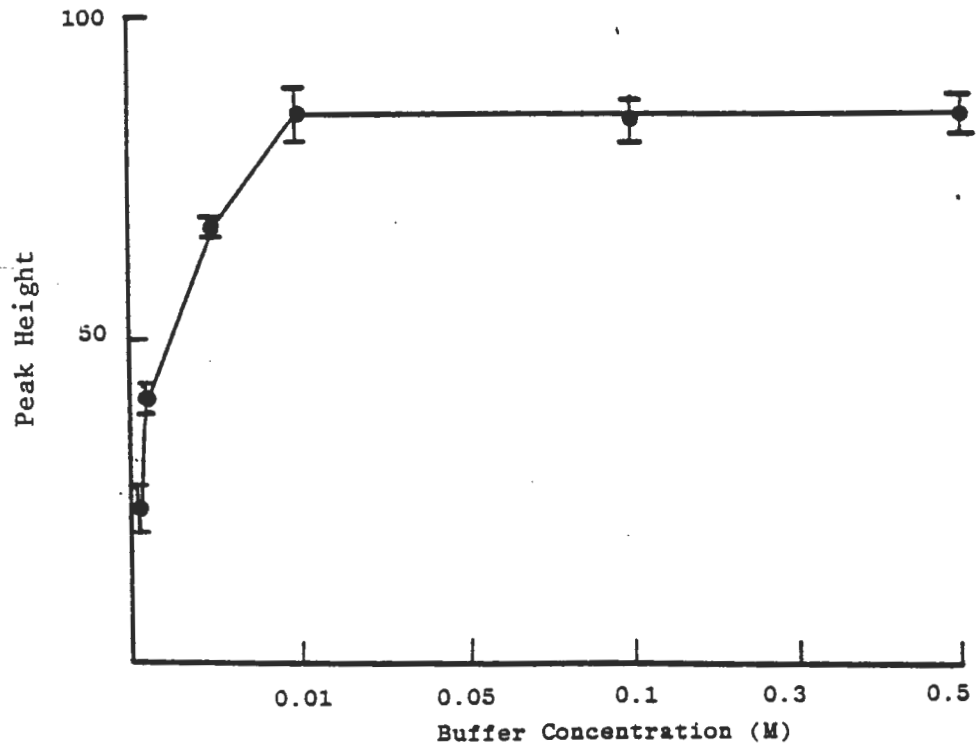
Figure 12: Effect of Buffer Concentration on Leucovorin
           Peak Height
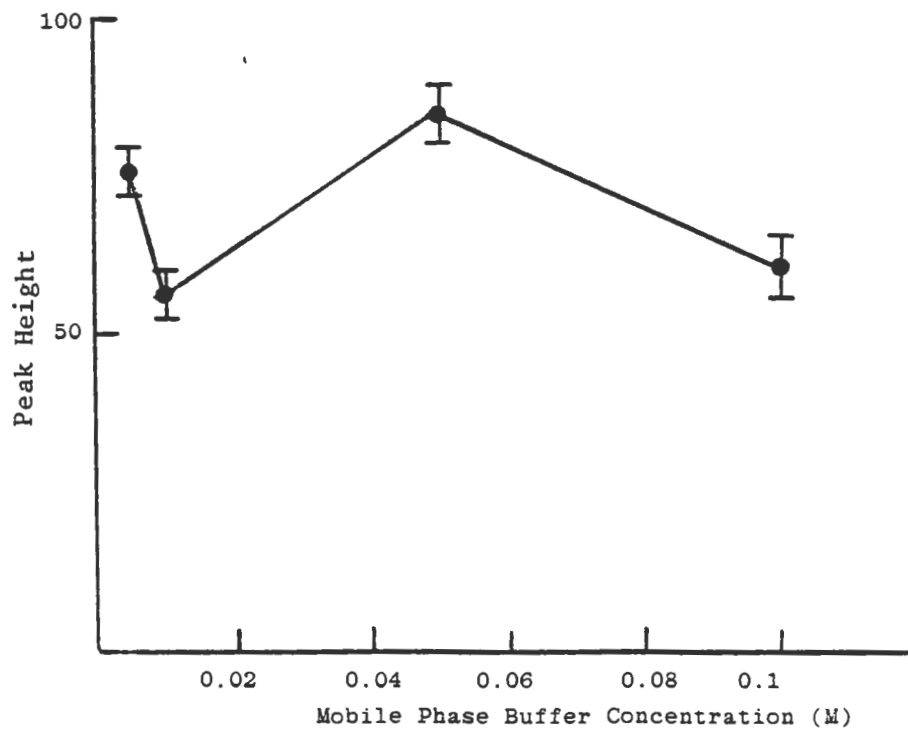
Figure 13: Effect of Buffer Concentration on Methyl-
tetrahydrofolate Peak Height

to a plateau value as was expected, the response of METHF described no discernible pattern. As the salt concentration rose, the response first decreased, then increased and finally decreased to a level lower than the initial salt concentration. This result was unexpected and cannot be explained readily.

v. Effect of Methanol on Detector Response

The elution of the reduced folates from the Sep-Pak minicolumnns required a higher methanol concentration than was present in the mobile phase, e.g., 50% versus 4%. Reece and Cozamanis (1979) have reported that samples dissolved in methanol show decreased detector response and poor resolution. They attributed the effect to the high local concentration of organic solvent altering the peak shape and causing band broadening. The effect is particularly pronounced with sample loops larger than fifty to 100 microliters and are used for sample loading. The effect of methanol on detector response to LEU and METHF was studied by preparing drug standards in solutions containing varying concentrations of methanol in 0.5 M ammonium phosphate pH 5.5. The electrochemical detector was set at +0.8 V applied potential; the summing amplifier was set at 50 nanoamps per volt; and the filter gain was set at 10. The chromatographic conditions for this

experiment were: MPII; with a flow rate of 3.0 ml. per minute; and the column temperature was 40°C.

Figure 14 represents peak height as a function of methanol concentration for LEU and METHF. One way ANOVA showed a significant methanol effect ($p < 0.05$) on peak height for both compounds. The effect of methanol was more pronounced on LEU than was the case for METHF. The effect of methanol on LEU can best be described by a straight line ($r^2 = 0.94$, n=18), METHF response is best described by some curvilinear function (r^2, = 0.92, n=18). This may be explained by the fact that METHF is apparently a more easily oxidized compound that LEU and, as such, is less subject to variation in response. Contrary to the findings of Reece and Cozaminis (1979), no significant difference in peak width (or resolution) was noted with increasing methanol concentration. Although a high methanol content in injected samples is clearly not desireable, the final organic strength was dictated by the eluent which removed the folates from the minicolumn most efficiently.

B. Chromatography

Reverse-phase chromatographic methods are estimated to account for 60% to 70% of all analytical work performed using HPLC (Horvath and McLean, 1977). A review of the recent pharmaceutical and chemical literature shows that reversed-phase systems, particularly those which use microparticulate octadecyl-, octyl-, or phenyl silica
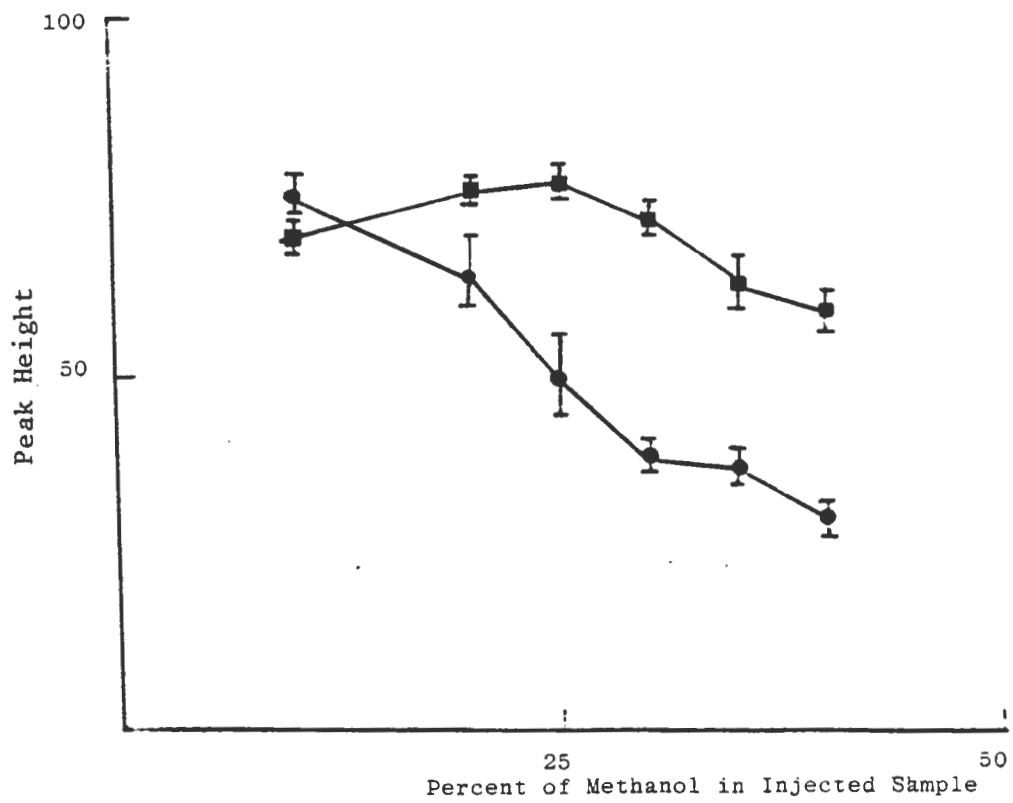
Figure 14: Effect of Methanol on Detector Response for
Leucovorin and Methyltetrahydrofolate

stationary phases, are becoming increasingly popular for the analysis of drugs.

1. C-18 Stainless Steel Column

A microparticulate octadecyl-silica stationary phase was chosen for evaluation for three reasons; (1) MTX, structurally related to LEU, has been successfully chromatographed using this system, (2) stationary phases having high carbon content, i.e., octadecyl vs octyl-silica are reported to have a large intrinsic retentive capacity (Horvath and McLander,1977) and (3) there is a large body of information available regarding drug analyses employing this stationary phase.

Because the reduced folates to be chromatographed are amphoteric compounds, the use of paired-ion reagents was considered necessary. Gloor and Johnson (1977), reported that for strong and weak acids, typical counter-ions such as quaternary amines (tetrabutylammonium chloride for example) are generally used and, that for strong and weak bases alkyl and aryl sulfonates, such as pentane, heptane, and octane sulfonic acids are among the best choices. The paired-ion reagents best suited for use with amphoteric compounds is however, less well defined. It has been suggested (Rausch, 1977) that sulfonic acids may provide the best separation of amphoteric compounds similar in structure to the reduced folates.

The most commonly employed solvent systems in

reversed-phase paired-ion chromatography are water/methanol or water/acetonitrile combinations. Therefore, the first mobile phases evaluated were combinations of methanol and water with 5mM PIC B5, (pentane sulfonic acid, Waters Assoc.). Figure 15 is the chromatogram resulting from the separation and detection of 1×10^{-6}M LEU in a mobile phase consisting of 25% MeOH: 75% Pic B5, p3.5. The retention of LEU was found to be 7.25 minutes. The peak however, was broad, and eluted over a prolonged, 4 minute period. In addition the system was not suitably sensitive, as is evident from the small peak obtained for LEU.

Various combinations of paired-ion reagents, organic solvents, and pH's were prepared and evaluated. Three factors were considered in the evaluation of a mobile phase; capacity, selectivity and sensitivity for the reduced folates.

Capacity may be calculated according to Equation xx and may be defined as the ability of a chromatographic system to retain a compound.

$$k' = \frac{V_t - V_o}{V_o}$$

(Equation 18)

Where Vt is the retention volume (or time) of the compound of interest, and Vo is the void volume or volume in which a non-retained compound would elute.

Selectivity is the ratio of 2 capacity factors and
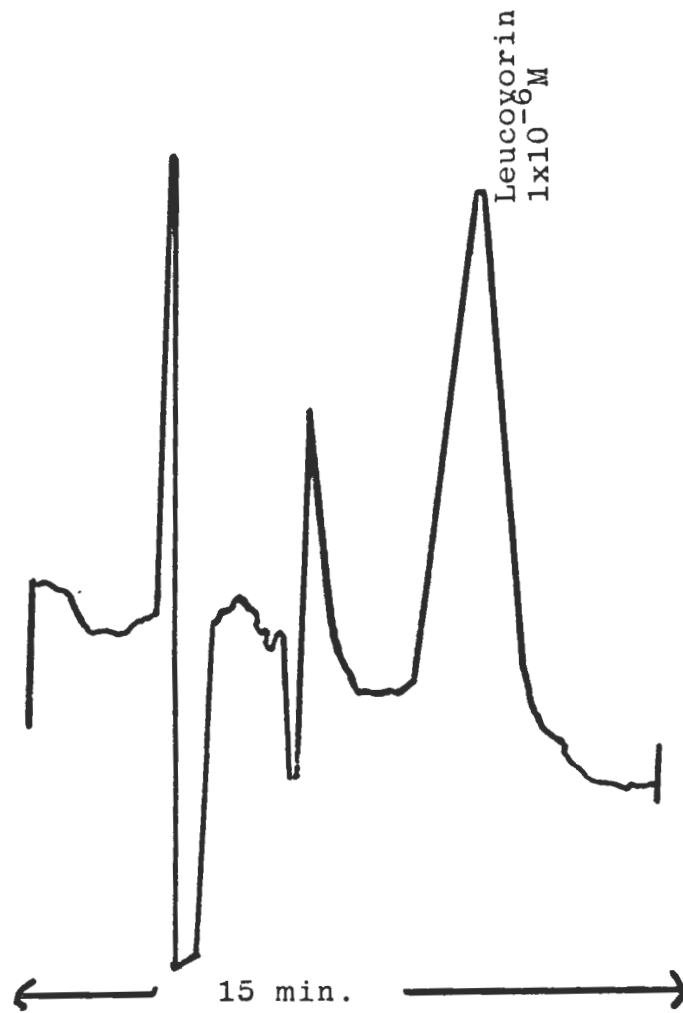
Figure 15:   Leucovorin Chromatogram
             using Paired-Ion Reagent B_7

describes the ability of the system to separate two compounds.

Table III shows representative mobile phase compositions used with the microparticulate C-18 column, and the resulting capacities and selectivities which were obtained. Figure 16 is the chromatogram of what was considered the optimum separation obtained from the reduced folate analogs which could be detected. In general the capacities calculated for LEU were relatively low, while those obtained for METHF considerably larger.

The addition of aqueous buffer to some of the mobile phase systems described in Table III was the result of the poor sensitivity obtained from systems containing only paired-ion reagents. Such systems have relatively high resistance, and as a result the current produced from the oxidation of a sample is low. The increased ionic strength of mobile phases containing the various buffers increased sensitivity but, in general, had a negative effect on capacities, resolution, and efficiencies.

A reported advantage of reversed-phase paired-ion chromatography is the ability to make direct injection of biological fluids without prior extraction or sample "clean-up" (Johansson et al. 1979). This, however, was not possible under the chromatographic conditions employed. Plasma or serum injected directly onto columns produced chromatograms of (presumably) polar compounds eluting for longer than 20 minutes, and less polar compounds still

TABLE III

REPRESENTATIVE MOBILE PHASE COMPOSITIONS

USED WITH REVERSE-PHASE C-18

STAINLESS STEEL COLUMNS

Mobile Phase	Retention Time (Min.)	
	LEU	METHF
(1) Octane Sulfonic Acid: CH_3OH: CH_3CN; 70: 25: 5; pH 3.5	7.5	26.5
(2) Octane Sulfonic Acid: CH_3OH: CH_3CN; 70: 22: 8; pH 3.5	7.5	20.5
(3) Acetic Acid: Sodium Acetate 0.1M: CH_3CN; 75: 25; pH 4.5	10.0	10.0
(4) Heptane Sulfonic Acid: CH_3OH: CH_3CN; 82: 15: 3; pH 3.0	12.9	20.0
(5) Heptane Sulfonic Acid: .01M in KH_2PO4: CH_3OH: CH_3CN; 80: 17: 3 pH 3.0	17.07	8.23
(6) $(NH_4)_2HPO_4$ 50mM: CH_3OH: CH_3CN; 80: 17: 3; pH 6.5	4.52	3.75
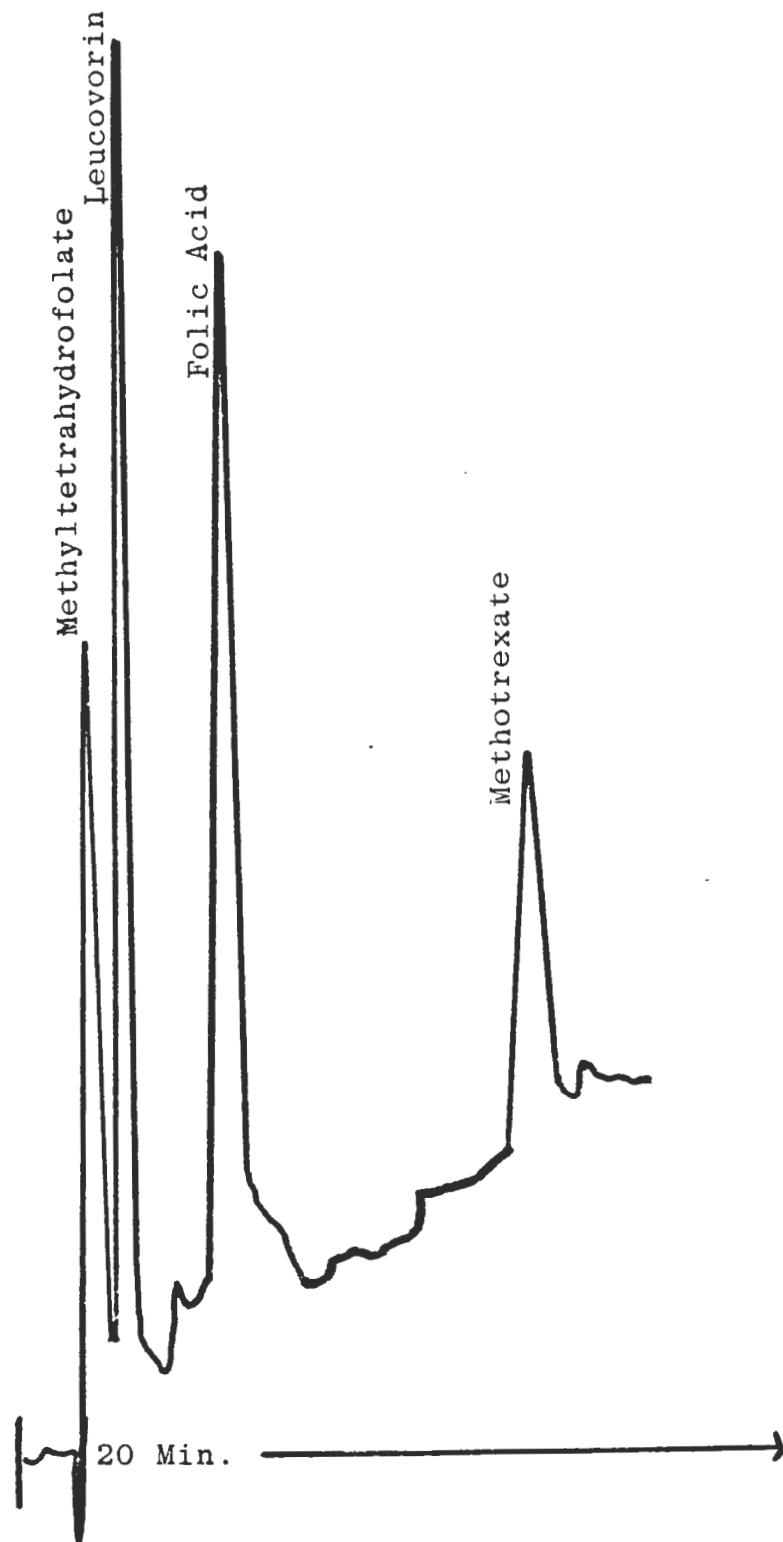
Figure 16:   Resolution of Folate Analogs Using
C-18 Stainless Steel Column

evident 60 minutes after injection. Similar reports of interfering compounds adversely effecting electrochemical detectors have been published by Kissinger (1974) and Lankelma and Van der Kleijn (1978).

It was not possible to devise a separation system using the C-18 column which could suitably separate the reduced folates from plasma constituents.

2. Radial Compression Separation System

A recent advance in analytical liquid chromatographic technology is the development of radial compression column systems. Radial compression has been reported to have several advantages over conventional stainless steel column technology including: high efficiency at higher flow rates, increased resolution, greater column to column reproducibility, low system operating back-pressures, and longer column life. The operation of this system and its advantages have been reviewed recently by Fallick and Rausch (1979) and Little et al. (1976).

a. Evaluation of the Radial Compression

A Radial Compression Separation System (RCSS) was evaluated for its applicability to the separation of reduced folates in aqueous systems and serum. The column used for this purpose was an octadecyl-silane reverse-phase materal with an 8mm I.D. and a 3.0 ml internal volume.

Dibasic ammonium phosphate, $(NH4)2HPO4$, was considered

the most promising buffer, with respect to resolution and sensitivity achieved on C-18 packing material, and was the paired-ion agent first used in evaluating RCSS technology.

Chromatographic conditions suitable for conventional reversed-phase separations may not be directly applicable to the RCSS technology. Decreasing the organic solvent strength by approximately one-half of that in a conventional system has been recommended as a means of obtaining a suitable mobile phase from which to experiment. Because of the reported differences in performance between the two chromatographic methods and the paucity of published data regarding its operation, a characterization of several RCSS operating parameters, with respect to reduced folates, was undertaken. The four characteristics used to evaluate the RCSS were: mobile phase flow rate; column temperature; buffer strength; and pH.

i. Effects of Flow Rate

The large internal volume and shorter length of the radial pak cartridge, in comparison to conventional stainless steel columns, results in lower operating pressures for any given flow rate. As a result, it was possible to operate the RCSS at flow rates as high as 6.0 ml min^{-1} without exceeding back-pressures of 2000 psi. Figure 17 is a plot of the retention times as a function of flow rate for both LEU and METHF. The mobile phase used in these determinations was 0.5M (NH4)2HPO4 93%, CH3CN 3%,

CH3OH 4%, pH 5.5. Conventional chromatographic systems are limited to flow rates of 1.5 to 2.0 ml min^{-1}, higher rates exceed column back-pressures of 2000 psi and may damage the packing material. Over these limited solvent flow rates, plots of retention times as a function of flow rate are linear. However, when higher flow rates were used, deviations from linearity were evident. It is apparent from this figure that both the capacity and selectivity of the RCSS decreased with an increase in flow rate above 3.0 to 4.0 ml min^{-1}. Figure 18 is a log-log transformation of the retention times as a function of flow rate. The linear regression coefficients obtained from this plot were subsequently used to predict the retention of LEU and METHF during the development of optimal mobile phase conditions. It was possible to extrapolate this data from the mobile phase tested and predict retentions in other mobile phase systems as might be expected. These observations are in general agreement with studies of flow control in HPLC reported by Schrenker (1978).

ii. Effects of Temperature

"In HPLC there has been a tendency to overlook the influence of temperature on chromatographic measurements" (Gilpin and Sisco 1978). These authors report that both normal and bonded phases may be affected by temperature, and that variations in capacity greater than 25% have been observed with temperature changes of 1°C. or less. Because
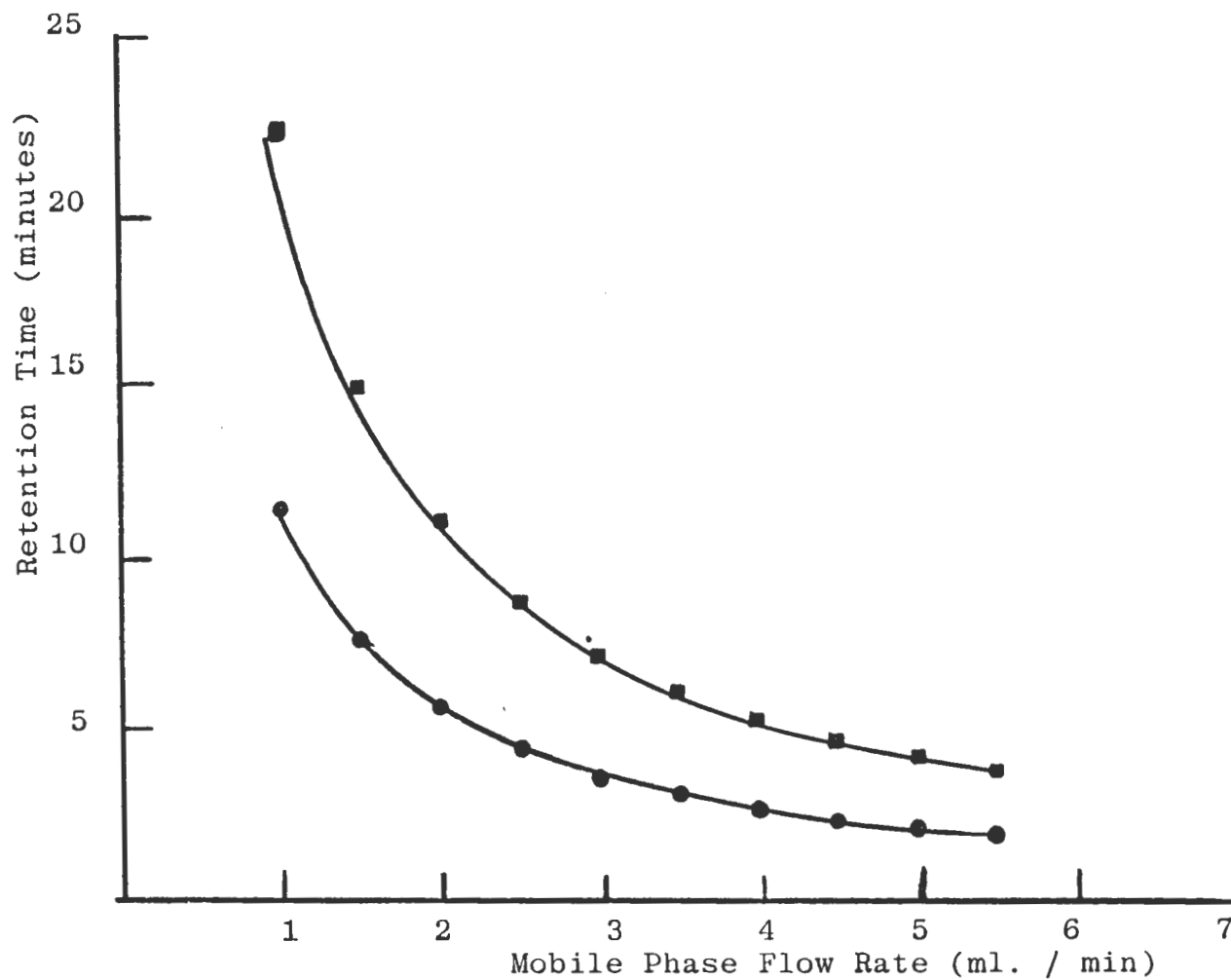
Figure 17: Effect of Mobile Phase Flow Rate on the Retention of Leucovorin ● and Methyl-tetrahydrofolate ■
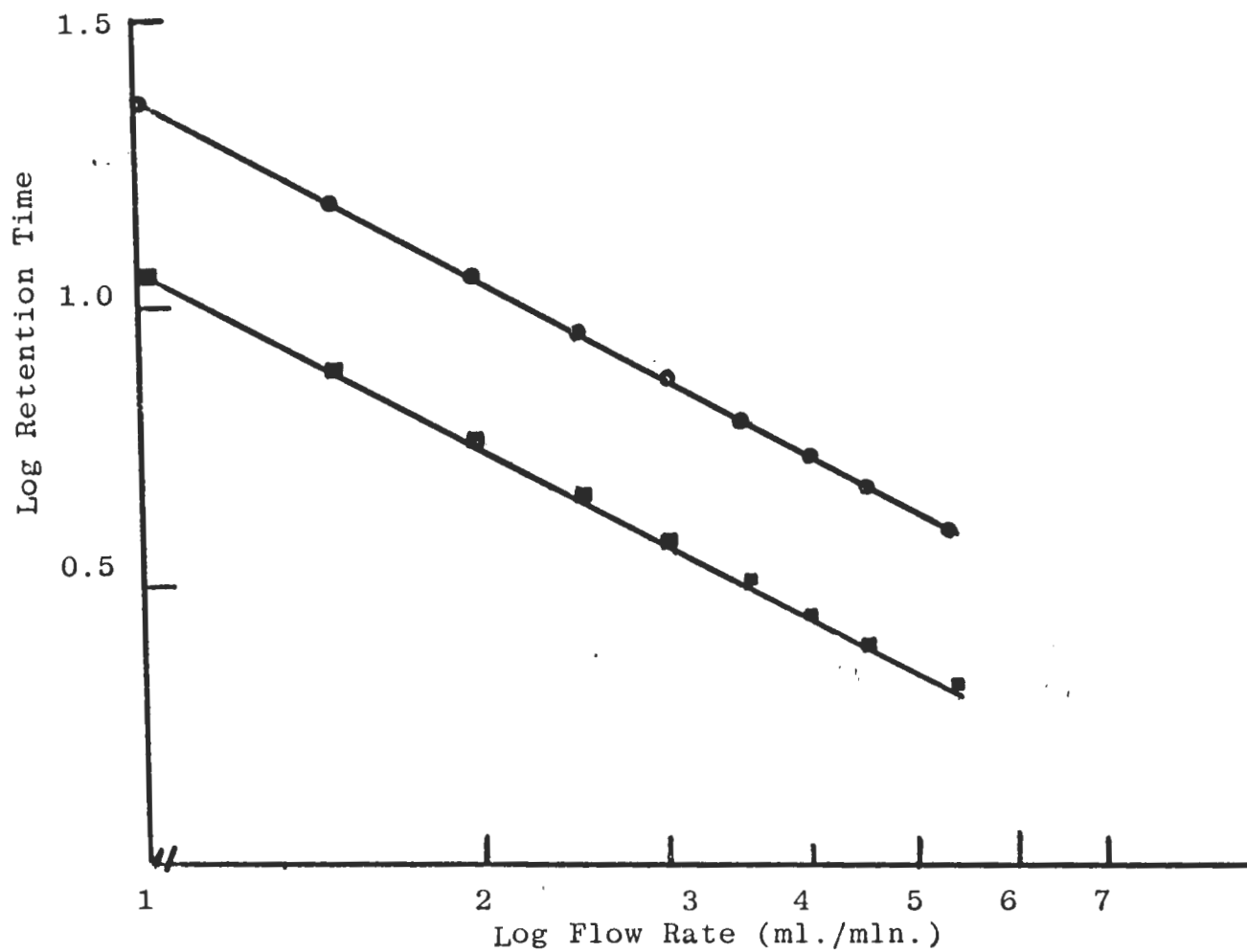
Figure 18: Log-Log Transformation of Retention Time as a Function of Flow Rate Leucovorin Methyltetrahydrofolate

of the high efficiency and reactivity associated with RCSS columns the effect of temperature on retention times of LEU and METHF was investigated.

Table IV is a comparison of LEU and METHF retention and the corresponding column selectivity calculated at 20°C, 30°C, 40°C, and 50°C. Figure 19 shows the chromatograms obtained from the injection of a LEU-METHF combination at 20°C and 50°C. The decrease in retention times (capacity) at the two temperatures is evident, however, the selectivity of the system actually improved at the higher temperature. This can best be explained by a decrease in both LEU and METHF peak width.

Temperature fluctuations were found to affect daily chromatographic results. Ambient temperatures fluctuated as much as 10°C. during the course of a day, causing as much as 20% changes in observed retention times for folate compounds using the same mobile phase. The installation of a Thelco, Model 15, constant temperature oven, circumvented the effects of temperature. Column temperatures were held constant at 40°C ± 3°C and retention times for LEU and METHF were found to be reproducible within the 5% limits of the RCSS system.

iii. Effects of Counter-Ion Strength

The effect of counter-ion (buffer) concentration on the retention of LEU and METHF was characterized during the evaluation of mobile phase characteristics.

TABLE IV

EFFECT OF TEMPERATURE

ON FOLATE RETENTION

Temperature ($^{\circ}$C)	Retention Time (Minutes)		Selectivity (α)
	LEU	5-METHYL	
20°	4.60	8.10	1.95
30°	4.13	7.52	1.89
40°	3.50	6.59	2.27
50°	2.68	4.90	2.36
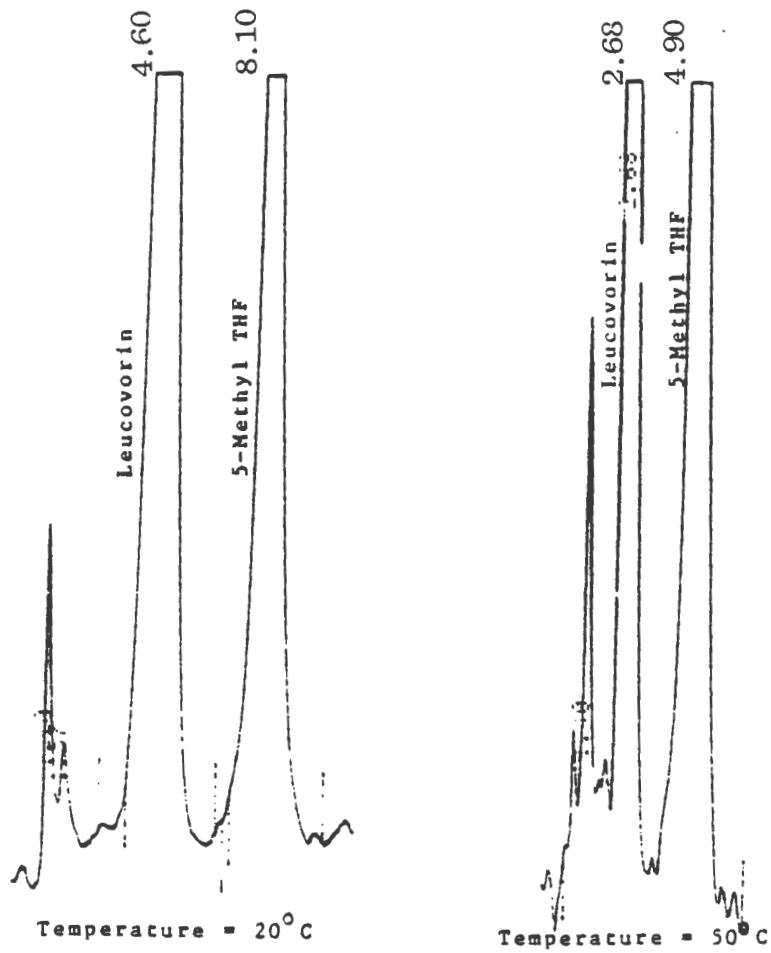
Figure 19: Effect of Column Temperature on Leucovorin and Methyltetrahydro-folate Retention.

The mobile phase contained Buffer:MeOH:CH3CN in the ratio 93:4:3. The pH was maintained at 5.5. Table V describes the retention times for LEU and METHF as a function of ammonium phosphate concentration. Although the mechanism of the ion-pair reaction is unclear (Gloor and Johnson, 1977) and is further clouded by the presence of positive and negative counter-ions from the ammonium phosphate, the general trend is in agreement with published data suggesting that increasing counter-ion concentration increases retention up to a limiting value.

iv. Effect of pH

The effect of pH is dependent upon the nature of the solute. However, retention increases as changes in pH tend to maximize the concentration of the ionic form of the solute. This presumably increases the equilibrium in favor of the ion-pair complex. Figure 20 is a plot of the retention of LEU and METHF as a function of pH. The mobile phase used was 0.5M Phosphate Buffer:CH3OH:ch3cn in the ratio 93:4:3. It is evident that pH affects LEU retention to a greater degree than it does METHF. This result however, is not easily explained. Although pKa values have not been reported for METHF it would seem likely that it would have values similar to those of LEU. Both compounds have the same ionizable groups, e.g. C4 hydroxyl group, and 2 glutamyl carboxyl groups. It would seem likely that the disassociation of these groups would be similar

TABLE V

THE EFFECT OF BUFFER CONCENTRATION

ON FOLATE RETENTION

	Retention Time (Min.)	
Buffer Molarity	LEU	METHF
0.5	3.85	7.49
0.3	3.79	6.76
0.1	2.91	6.06
0.05	2.62	5.66
0.01	1.63	3.66
0.005	1.57	2.06

Mobile Phase Composition:

Buffer: CH_3OH: CH_3CN; 92:4.5:3.5 pH 5.5

Flow Rate = 3.0 ml/min.
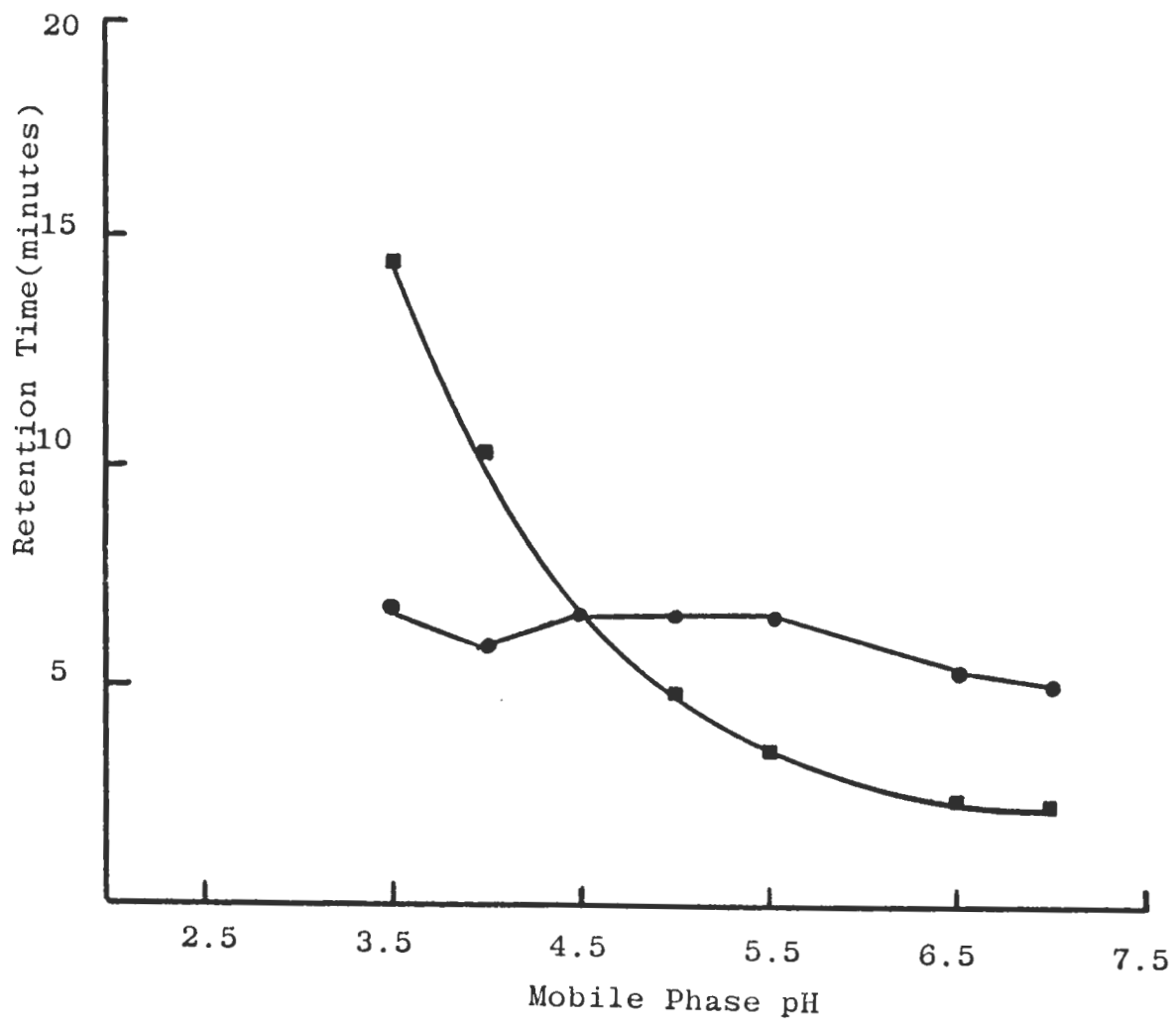
Figure 20: Effect of Mobile Phase pH on the Retention
of Leucovorin ■ and Methyltetrahydrofolate ●

for both compounds. Since the only structural difference between LEU and METHF is in the N^5 substituent, formyl and methyl respectively, these data suggest that the functional group in this position contributes to the retention characteristics of these reduced folate analogs.

C. Drug Assay

1. Aqueous Systems

The electrochemical detector response to LEU or METHF, measured as peak height, declined rapidly from a maximum value observed immediately after the cell was resurfaced. As a result, it was necessary to generate standard curves of peak height as a function of concentration, whenever quantitative measures were to be performed.

Calcium LEU or Barium METHF were used as received without further purification. LEU and METHF solutions of $1x10^{-3}M$ and $1x10^{-4}M$ respectively were freshly prepared in 1N KOH. All further dilutions were made with the solvent in which the drug was to be chromatographed; Sorenson's Buffer for protein binding (LEU or METHF) or, mobile phase for LEU serum concentrations. All drug solutions prepared in this manner were protected from light with aluminum foil and stored on ice. Fresh stock solutions were prepared daily. No degradation in either drug solution was noted; no detectable changes in peak heights of the chromatograms or by changes in the shape of peaks or by the appearance of other peaks or shoulders in the chromatograms during the

course of an experiment.

Standard curves for both LEU and METHF were generated by plotting peak height as a function of drug concentration. Drug concentrations for this purpose were between 2.5×10^{-7}M and 5.0×10^{-6}M. At least 5 concentrations were used to generate these standard curves. Each point represents the average of 2 determinations; each concentration was determined in triplicate. Thus, each standard curve consisted of at least 15 data points. A linear plot was obtained over the concentration range specified for both drugs ($r^2 > 0.99$ in all cases). All unknown solutions were analyzed in duplicate and concentrations were determined from the regressions coefficients of the appropriate standard curve.

2. Serum

A LEU stock solution, 1×10^{-3}M, in 1N KOH, was prepared, and from this stock solution, additions were made to pooled human serum to yield final LEU concentrations between 2.5×10^{-7}M and 2.5×10^{-6}M. The serum samples were then extracted in the manner described previously. All samples were analyzed the same day as extracted.

The evaporated serum samples were reconstituted in 0.5 ml of mobile phase. Since the extraction was carried out with 1.0 ml of serum, this represents a two-fold concentration of sample. Care was taken to rinse the walls of the sample dish to insure collection of the entire

sample.   Identity of the LEU peak in the sample matrix was confirmed by analyzing serum-blanks which exhibited no peaks with retention times corresponding to LEU, and by the addition of LEU to reconstituted samples and noting an increase in the height of the peak identified as LEU.

a. Recovery

The recovery of LEU added to pooled human serum was virtually complete.   The recovery over the concentration range studied was 101.11%, with a range in recoveries of 93.60% to 117.20%.   Table VI shows the amount of LEU added to serum samples, the calculated amount of LEU measured and the percent recovery at each concentration.   Figure 21 is a plot of the observed LEU concentration as a function of the known LEU concentration in each sample.   A plot of LEU peak height as a function of concentration was linear over the concentration range $2.5 \times 10^{-7}M$ to $2.5 \times 10^{-6}M$.   A statistical analysis of the regression coefficients obtained from standard curves of LEU in serum and mobile phase was performed.   No difference ($p < 0.05$) was noted in the slope of the regression.   The intercepts, however, showed a signficant difference ($p < 0.05$), those obtained from LEU in serum were larger than from aqueous systems.   Although the differences in y-axis intercepts were statistically different ($p < 0.05$), the error caused by using the regression coefficients obtained from LEU in mobile phase

TABLE VI

RECOVERY OF LEUCOVORIN FROM HUMAN SERUM

Initial Serum Concentration[a] (Moles/Liter)	Calculated Serum Concentration[a] (Moles/Liter) (Mean $\pm$ S.D.)	Average Percent Recovery +
2.5	2.34 $\pm$ 0.30	93.60
5.0	5.86 $\pm$ 0.60	117.20
7.5	7.50 $\pm$ 0.20	100.00
10.0	9.62 $\pm$ 0.60	96.20
15.0	14.20 $\pm$ 0.20	94.67
20.0	20.50 $\pm$ 5.10	102.50
25.0	25.90 $\pm$ 8.88	103.60

+ n=3

a = x 10^7
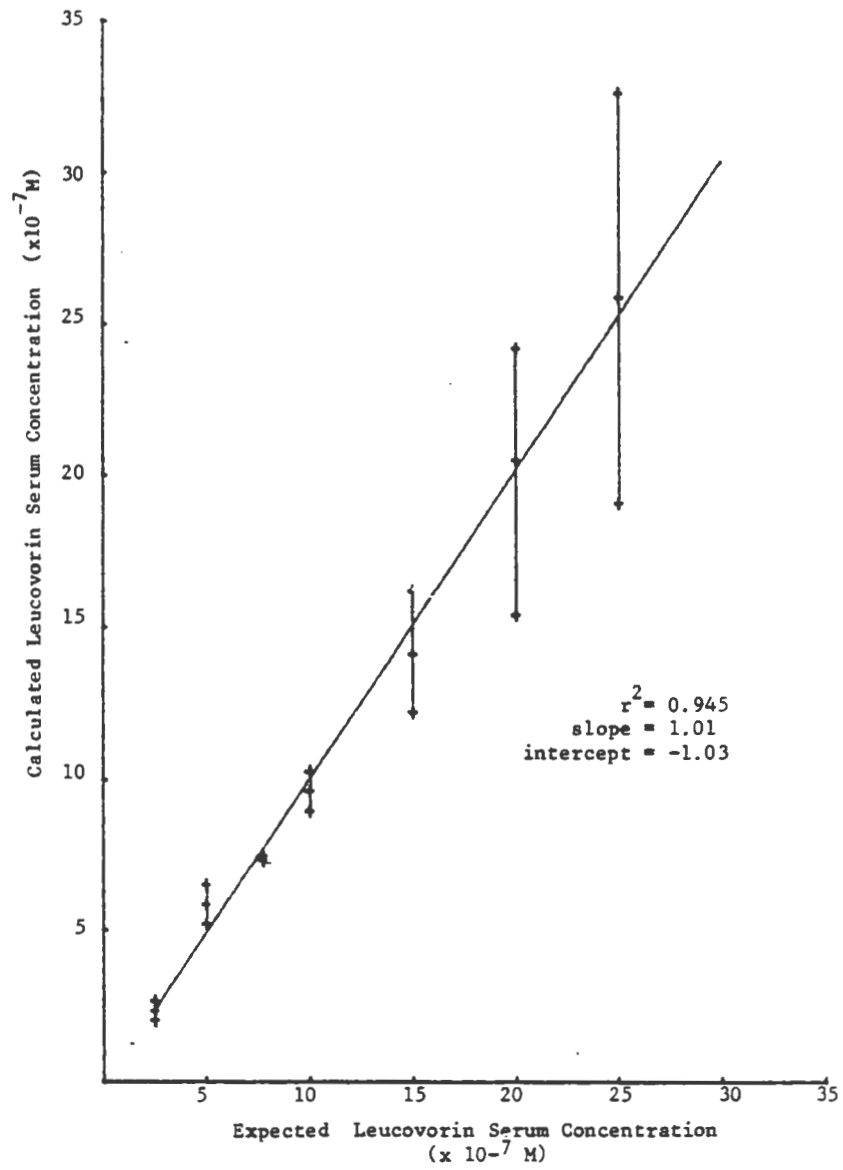
Figure 21: Leucovorin Recovery from Serum
Observed vs Expected Leucovorin
Concentration

to analyze serum samples were calculated to be less than 5 percent.

b. Sample Stability

The stability of LEU in the sampling dishes was evaluated in order to determine the maximum length of time that samples could be stored in this condition.

Leucovorin was added to 20 ml. of pooled human serum to yield a final concentration of 1.07×10^{-6}M. Nine, 1 ml serum samples were then extracted and evaporated as previously described. The samples were randomly assigned to 3 groups of 3 samples each, which were then frozen until assayed. The first group was assayed the day following extraction; the second, 2 days after extraction; and the third was assayed on the fifth day after extraction. The results of these assays were combined with the results of 3 assays performed immediately after the extraction process, and appear in Table VII.

The calculated LEU concentration following the extraction procedure was compared with the LEU concentration in the serum samples prior to extraction. No statistical differences were noted when samples were assayed the same day, one day, or two days following the extraction procedure (Duncans Multiple Range test, $p < 0.05$). However, when the samples were stored for 5 days, there was a significant decrease in the calculated LEU concentrations. From these data is was concluded that

although analysis of serum samples following the extraction procedure need not be done immediately, two days was the maximum acceptable time between the extraction and analysis of LEU serum samples. Serum samples from all cancer patients and all normal subjects were assayed the day of extraction or within 24 hours.

3. Separation of Leucovorin in Serum

Following the characterization of several of the important RCSS operating parameters, a mobile phase was developed which effectively separated LEU from the oxidizable, extracted serum components (Table III).

Figure 22 shows representative chromatograms for LEU and METHF, which were obtained using the parameters detailed in Table VIII. The capacities, selectivity, resolution, and efficiency of the chromatographic system is given in Table VIII.

The capacities calculated for LEU and METHF fall in the range which is generally considered to be optimal, i.e., 2 to 10.

Resolution is a means of measuring the degree of separation of any two components in a chromatogram and is usually defined as the difference between the peak centers of two peaks, divided by the average base width of the peaks (Equation 18).

$$r = (V_t^2 - V_t^1) / 0.5 * (w_1 + w_2)$$

(Equation 18).

TABLE VII

STABILITY OF FROZEN LEUCOVORIN EXTRACT

Days Frozen	Initial Concentration[a] (Moles/Liter)	Calculated Concentration After Reconstitution[*][a] (Mean $\pm$ S.D.)		
0	1.00	0.96	$\pm$	0.07
1	1.07	1.05	$\pm$	0.18
2	1.07	1.14	$\pm$	0.10
5	1.07	0.82	$\pm$	0.07

* n=3

a = x 10^6
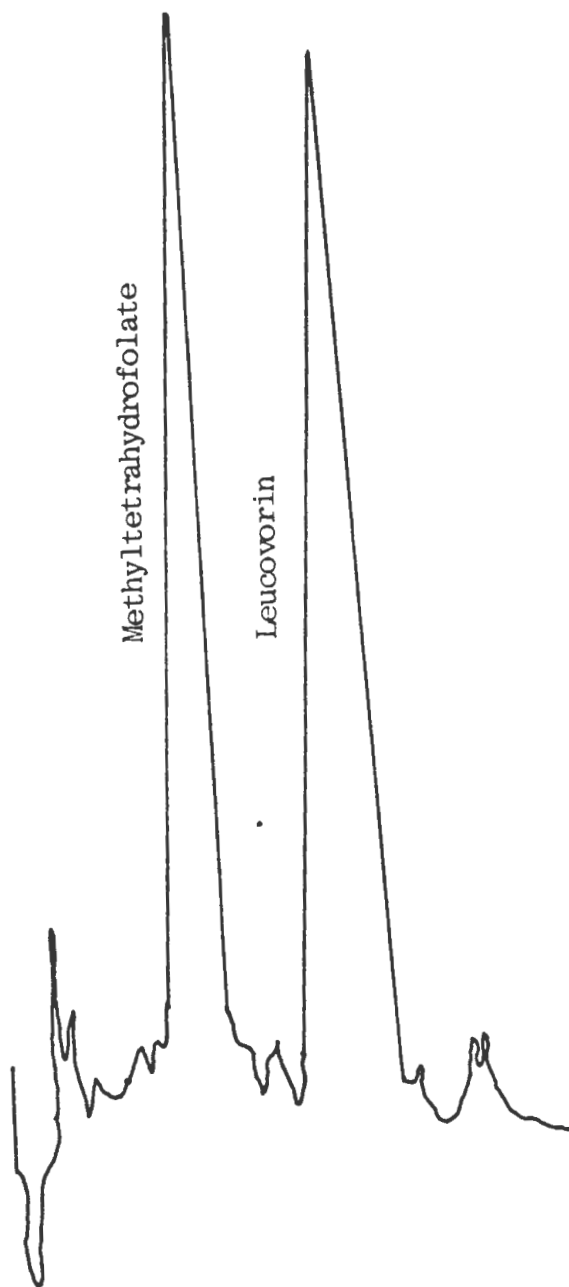
Figure 22: Separation of Leucovorin and
Methyltetrahydrofolate Using
Radially Compressed Column

TABLE VIII

CHROMATOGRAPHIC PARAMETERS DESCRIBING

THE SEPARATION OF LEUCOVORIN FROM

SERUM COMPONENTS

$(NH_4)_2$ HPO_4	92.00
Methanol	4.50
Acctonitrile	3.50
Sodium EDTA	10.00 MM
pH	3.50
Column: Radial Pak A	
Flow Rate: 3.0 ml/min	
Column Temperature: 40°C	
Leucovorin Retention	8.05 Min.
Leucovorin Capacity (K')	7.13
Resolution: (Leucovorin from the Closest Eluting Peak):	1.61
Column Efficiency (Leucovorin):	530.00 Plates

A resolution of 1.5 or greater indicates that two components are completely separated. The calculated resolution between the reduced folates was 2.07 indicating complete baseline resolution of these components.

Efficiency is the measure of band spreading of a peak throughout a chromatographic peak. High efficiencies generally produce sharper peaks than do low efficiency systems. The efficiency calculated for the reduced folates under the system employed in this work, is substantially lower than the manufacturers claim for the system (5000 plates). Chromatography of folate compounds generally results in low efficiencies which can best be explained by the amphoteric nature of the compounds. Because the compounds may exist in more than one ionic form during the separation, the relative retentions will differ. The net result of this effect is band broadening. A second explanation is the use of high buffer concentrations which are necessary for optimum electrochemical detection, and may adversely effect resolution and hence efficiency (Rausch, 1980).

Figure 23 is a chromatogram obtained from the injection of the reconstituted serum sample obtained from subject KB immediately before LEU administration. This chromatogram is representative of the 'serum-blank' obtained from all normal volunteers and patients who received MTX. Several peaks were found to elute before

LEU, and a single peak followed the drug. No additional peaks where observed when similar chromatograms were allowed to continue for 30 minutes. The serum peaks evident in Figure 23 were present in all samples chromatographed at approximately the same amounts in all extracted serum samples. These peaks, which were not identified, did not interfere with the quantification of LEU, although METHF, was masked with the unknown peak having a retention time of 4.6 minutes (versus 4.37 minutes for METHF)

Figure 24 shows the presence of LEU after administration of a 10 mg. intravenous dose to subject KB. This chromatogram was taken from the volunteers 6 hour serum sample. The resolution between LEU and the unidentified peak eluting at 6.12 minutes was calculated from this chromatogram. The resolution of 1.61 indicates complete resolution of LEU from the closest unknown peak in extracted serum samples. When the resolution between these two peaks declined to 1.25 or less, the chromatographic column was regenerated or replaced as necessary.
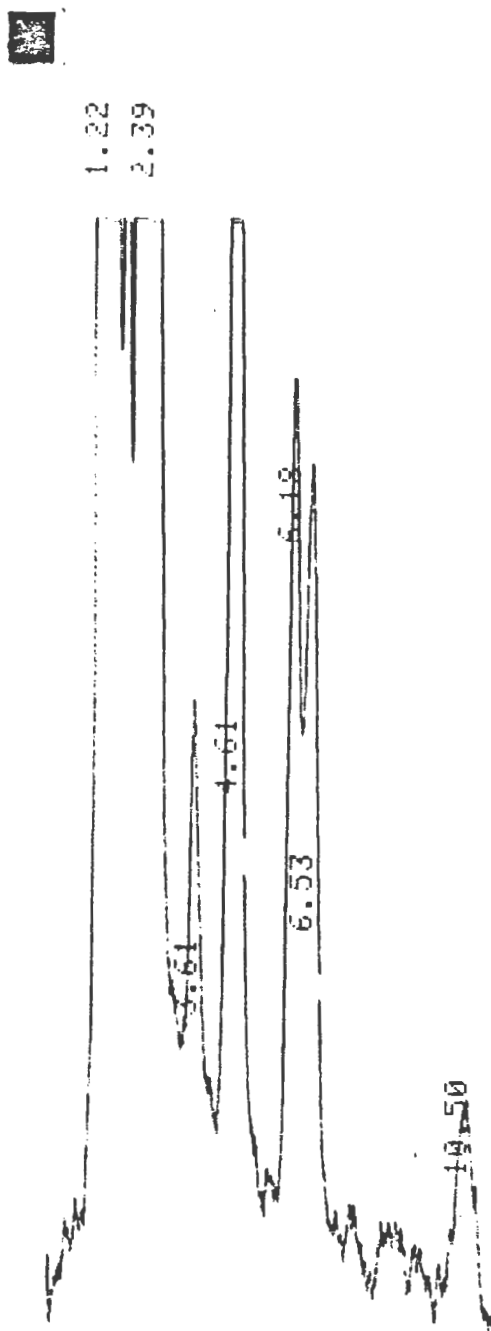
Figure 23: Serum Blank; Subject KB
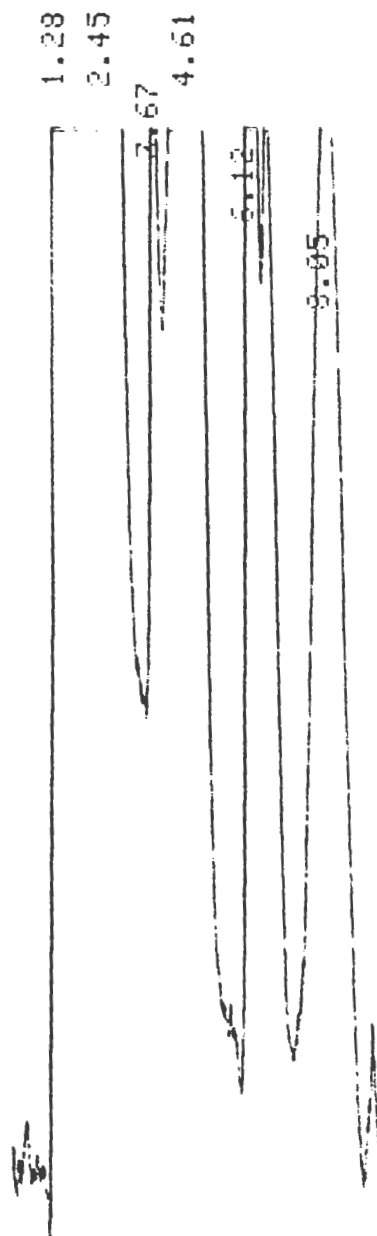
Figure 24:   Leucovorin in Serum;
             Subject KB

D. Protein Binding

1. Non-Specific Binding

Non-specific binding of either LEU or METHF to the components of the ultrafiltration cell or membrane was negligible. Filtered samples of the drug solutions showed no significant difference (Student's t, $p < 0.05$) when compared to samples taken from the ultrafiltration cell before being filtered.

2. Analysis of Albumin Solution

Samples obtained from the direct ultrafiltration of both dialyzed and non-dialyzed albumin were chromatographed. The samples obtained from the non-dialyzed albumin were shown to have a component or components which interfered with the quantification of both leucovorin and 5-methyl THF. The major interfering peak had a retention of 5.69 minutes and produced an input signal greater than 2.0 volts. Chloroform, toluol, and decanol, which may be used to induce crystallization of albumin, have been implicated as possible contaminants of commercially prepared albumin (Chignell, 1977). Dialysis of the albumin solution overnight at $4^{\circ}C$ against a large volume of water or buffer has been suggested as a means of removing such contaminants (Chignell, 1977).

Albumin used for the protein binding determinations was obtained from one source (Calbiochem Inc., Palo Alto,

CA.). However, two different lots of albumin were received. Lot No. 600638, used for the first two leucovorin binding experiments, was not dialyzed and nothing in the filtrate interfered with the drug analysis. Fraction V Human Serum Albumin Lot No. 903635, used for the final leucovorin binding study produced several peaks which interfered with the chromatography of both leucovorin and METHF. Dialysis successfully removed the contaminant(s) and no difference in LEU protein binding was observed using either dialyzed or non-dialyzed albumin.

Calbiochem Inc. confirmed (1980) that two different manufacturing processes had been used to prepare the two lots of albumin. Although the methods of preparation were not revealed, it seems likely that contaminant(s) such as those described by Chignell (1977) were present. The fact that dialysis of the protein solution readily removed the contaminants responsible for the interfering peaks is further evidence for this explanation.

3. Leucovorin Protein Binding

The protein binding of leucovorin was determined over a total drug concentration range of $5 \times 10^7 M$ to $1 \times 10^{-3} M$. The lower limit of binding determination was governed by assay sensitivity, the upper limit by leucovorin solubility.

Leucovorin binding to human serum albumin is essentially constant, $69.95\% \pm 4.70\%$, over the clinically significant concentration range. Figure 25 shows the

percent of leucovorin bound plotted as a function of total drug concentration. It is apparent from this figure that saturation of the protein had not occurred at the highest drug concentration examined. Moreover, binding did not decline appreciably with increasing drug concentration.

The limited solubility of leucovorin prevented the determination of binding at higher concentrations and also prevented the calculation of Scatchard parameters for LEU. Figure 26 represents a Scatchard plot for LEU. A set of data is considered admissible for Scatchard analysis if it meets the criterion of having an "everywhere negative slope" (Fletcher, 1973). The degree cf scatter evident in Figure 26 prevents the conclusion that this condition has been met. Therefore, the Scatchard model is not applicable for analysis of these data.

Values for affinity constants and numbers of binding sites can be estimated by stepwise computer analysis using iterative techniques (Fletcher, 1973). These techniques rely on reasonably accurate starting estimates, which are usually obtained by graphical methods. Such estimates are not available either by Scatchard, Scott, or Klotz plots because of the scatter in the leucovorin binding data and the limited range over which binding was determined.

4. Leucovorin Binding to Bovine Albumin

Albumin from various animal species can vary greatly in their drug binding characteristics (King, 1973).
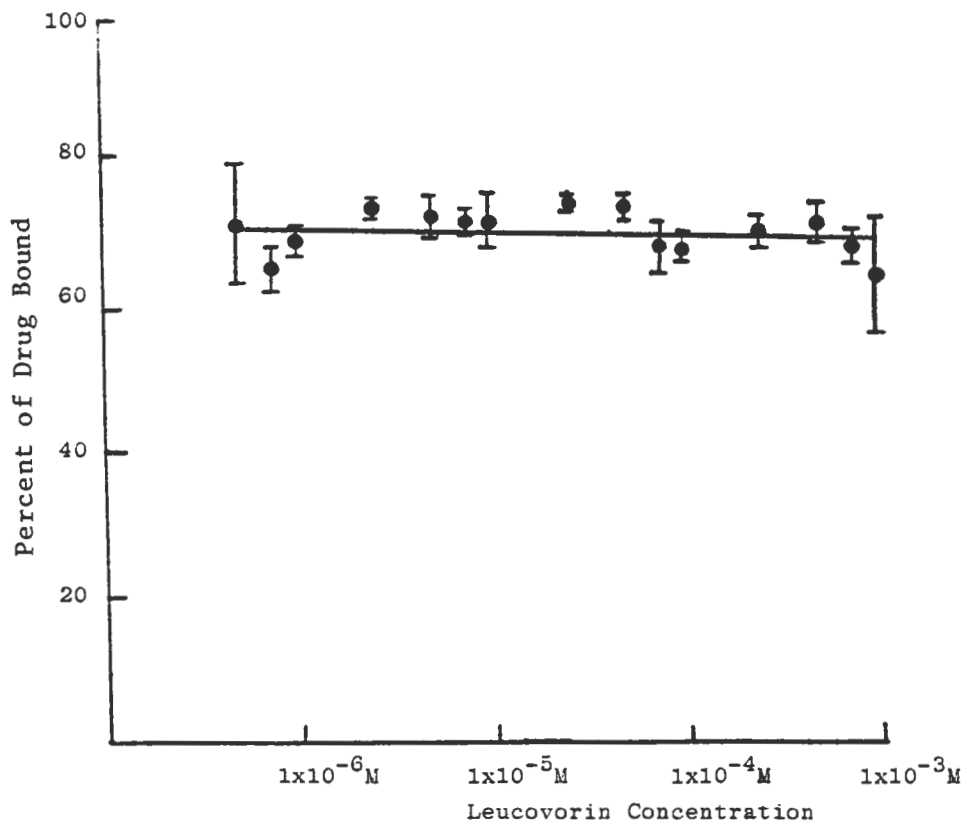
Figure 25: Percent of Leucovorin Bound to Human Serum Albumin as a Function of Concentration
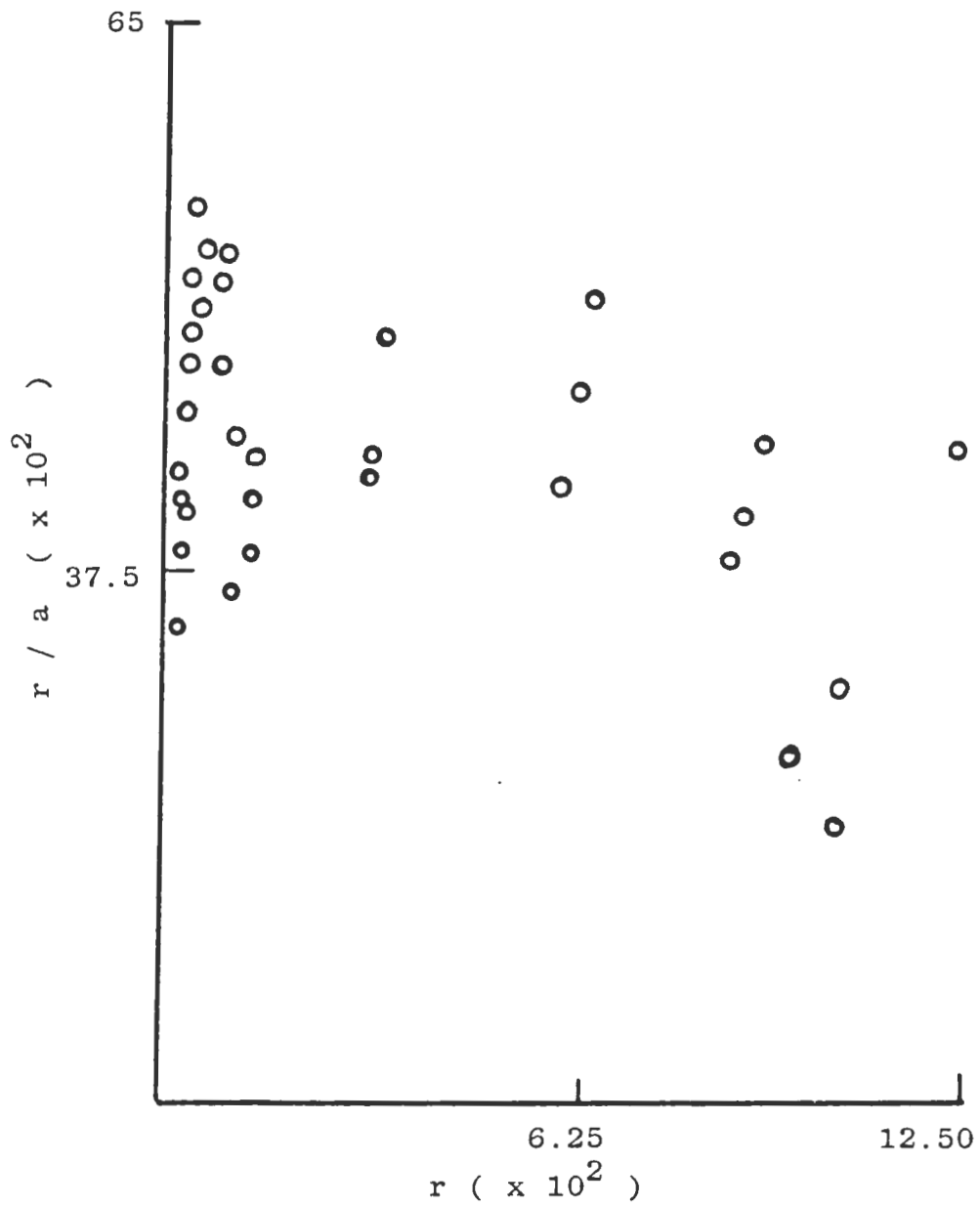
Figure 26: Scatchard Plot for Leucovorin
(r/a as a function of r)

Leucovorin binding to four percent (w/w) bovine serum albumin was determined over a drug concentration range of 2.5×10^{-6} M to 1.0×10^{-4} M, Figure 27. The binding was constant over the range studied ($69.37\% \pm 8.67$) as was the case with human serum albumin. A two way ANOVA was performed on these data, and it showed that no significant difference ($p<0.05$) existed between leucovorin binding to the two types of albumin.

5. METHF Protein Binding

METHF protein binding was determined over the drug concentration range of 5×10^{-7} M to 1×10^{-3} M. The binding of METHF, unlike leucovorin, did not remain constant. Figure 28 is a plot of percent drug bound as a function of total drug concentration. While saturation of the protein does not appear to have been reached, it is clear that the percent of drug bound to albumin decreased appreciably with increasing concentration. At 5×10^{-7} M, 86.24% of the drug was bound, at 1×10^{-3} M binding had decreased to 64.11%. In other words, the percent of free drug had increased 2.5 times over this concentration range.

Figure 29 is a Scatchard plot for METHF. As with leucovorin, the scatter in the data makes it impossible to assume that the criterion of an "everywhere negative slope" has been met. These data cannot be described in terms of a Scatchard model. Moreover, graphical techniques cannot yield reliable first estimates of Ka's or n's, and no final
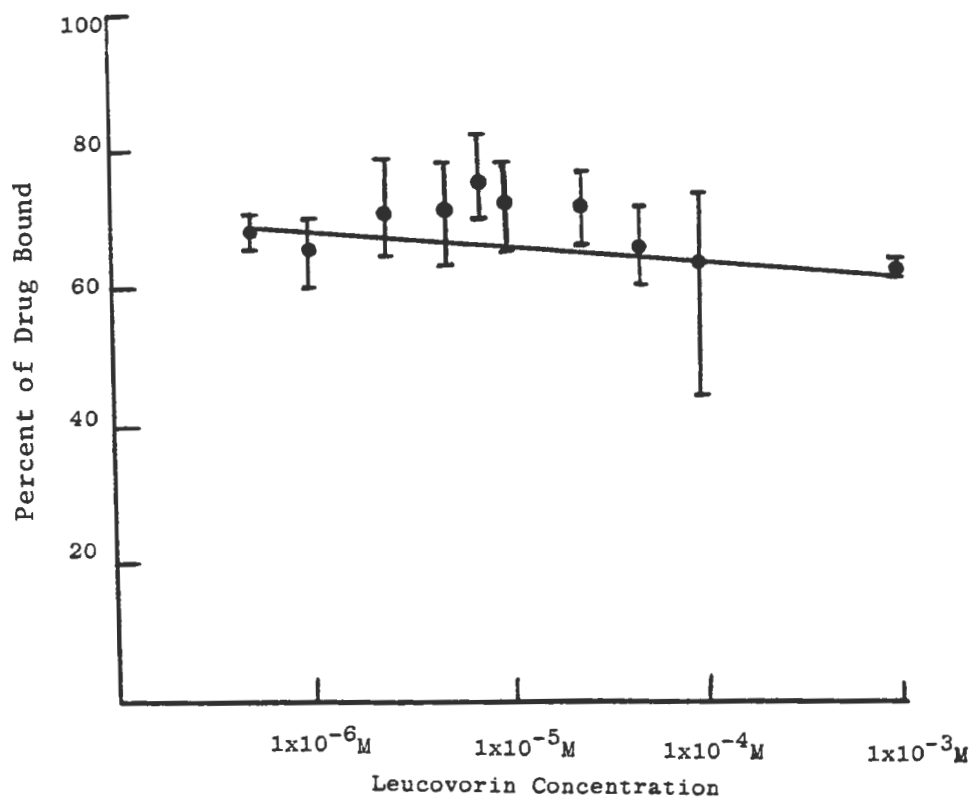
Figure 27: Percent of Leucovorin Bound to Bovine Serum
Albumin as a Function of Concentration
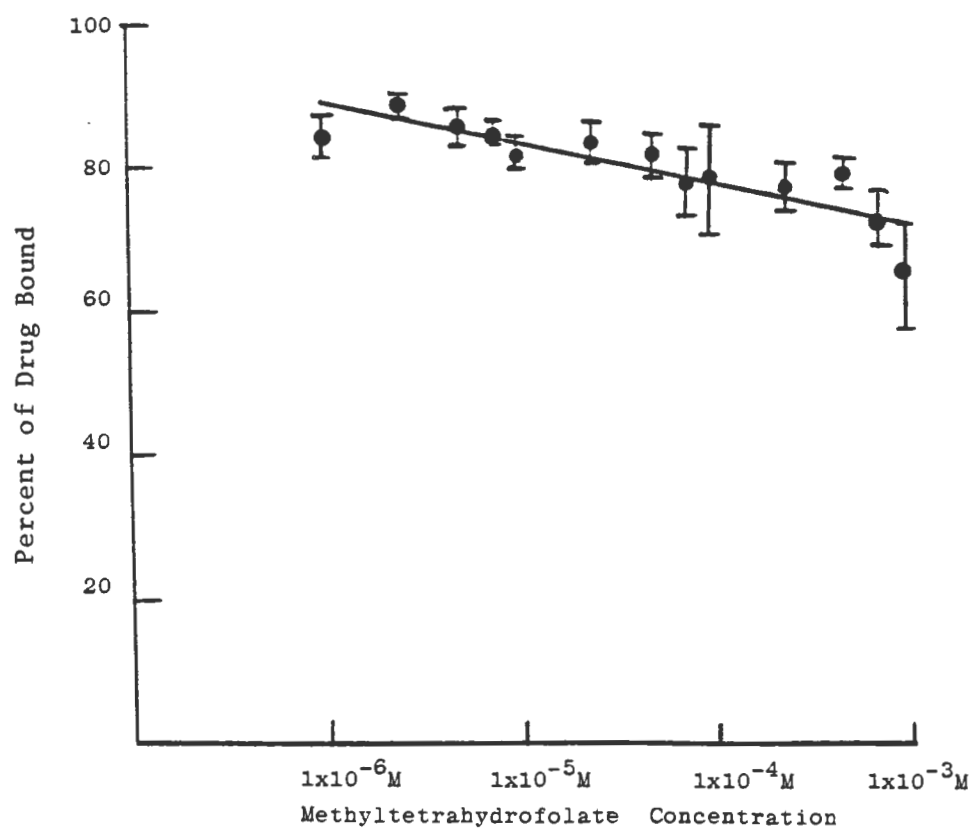
Figure 28:   Percent of Methyltetrahydrofolate Bound to Human
Serum Albumin as a Function of Concentration
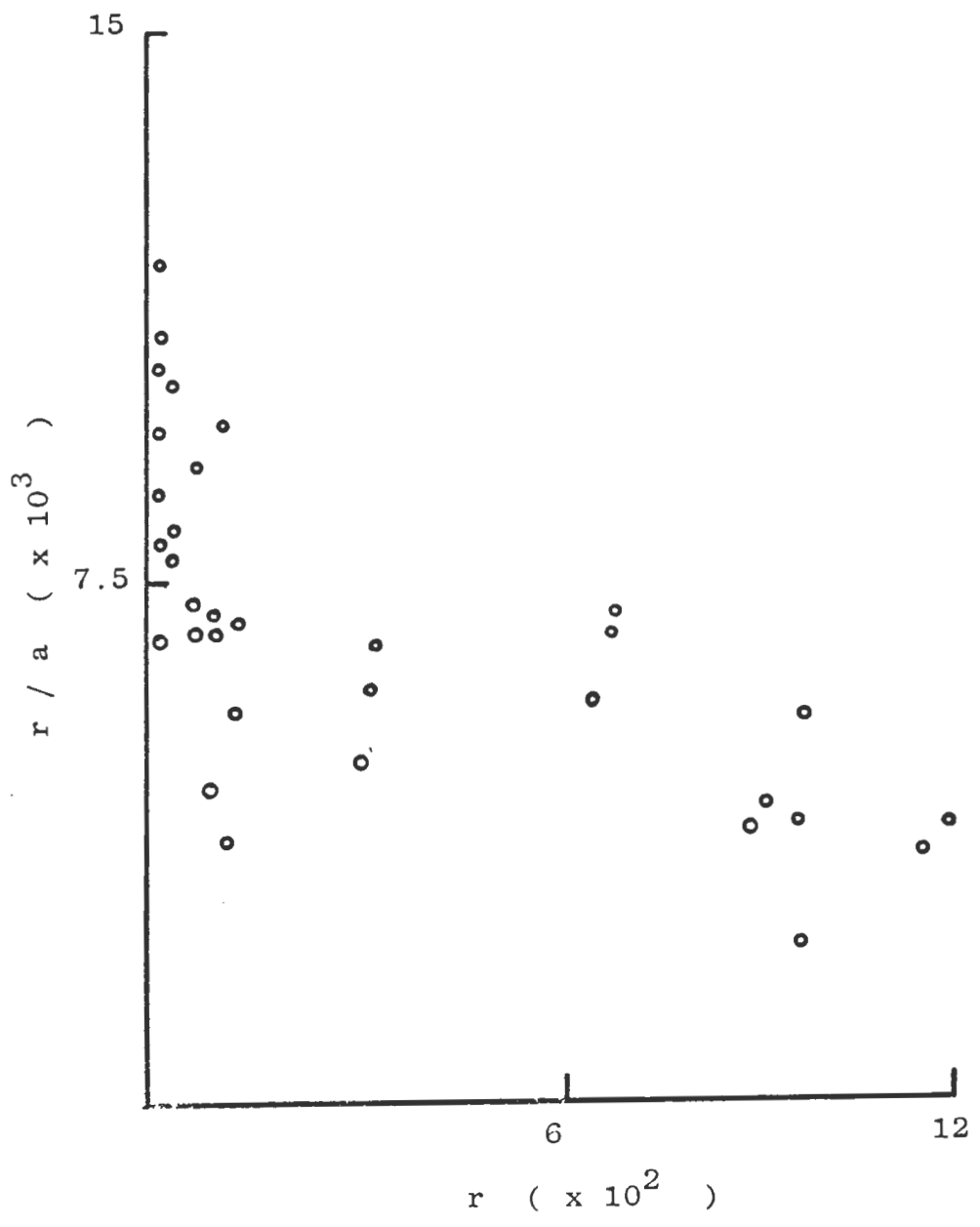
Figure 29: Scatchard Plot (r/a vs. r) for
            Methyltetrahydrofolate

values for affinity constants or numbers of binding sites can be obtained.

6. LEU, METHF Protein Binding Interactions

Since LEU and METHF coexist in plasma, there is the possibility of competition for a common binding site, which may alter the binding characteristics of one or both drugs. To investigate this potential interaction, the two drugs were ultrafiltered together and the protein binding determined. Four combinations of drug concentrations were evaluated: first, equimolar concentrations of LEU and METHF (5×10^{-5} M and 5×10^{-6} M); in addition, LEU was prepared in excess of METHF (5×10^{-5} M and 5×10^{-6} M, respectively); the concentrations were then reversed so that METHF was in excess.

The effect of METHF on the binding of LEU is shown in Table IX. The decrease in LEU binding in combination with METHF was significant at all levels tested (two way ANOVA, $p < 0.05$). The greatest change ocurred with METHF present in ten fold excess of LEU. The percent of LEU bound to albumin decreased from a mean value of $72.49\% \pm 2.62\%$ to a mean of $31.36\% \pm 4.94\%$.

The same result was obtained when the effect of LEU on the binding of METHF was examined. A significant decrease in METHF binding is observed with all combinations of LEU, Table IX. The greatest decline in binding, approximately 20, is observed when both drugs are combined at 5×10^{-5} M,

in comparison to METHF alone at this concentration.

The study of the protein binding interactions between LEU and METHF yields several conclusions. First, it is apparent that, since the degree of binding decreases for both drugs when they are present simultaneously, LEU and METHF appear to share a common binding site (or sites). Secondly, since METHF binding is influenced to a lesser degree by LEU than is LEU by METHF, it is reasonable to conclude that METHF has a stronger affinity for the site than does LEU. Finally, it is evident that determining the protein binding of any of the reduced folates (or folic acid) alone _in vitro_ does not reflect the physiological state in which folates exist. The differences in the degree of folate binding reported in the literature, particularly with respect to folic acid, may be attributable to the presence (or absence) of other folate forms at the time of assay, as well as to the method used to determine binding.

TABLE IX

LEUCOVORIN - 5-METHYL THF

PROTEIN-BINDING INTERACTIONS

LEU CONCENTRATION	METHF CONCENTRATION	
	$5\times10^{-6}M$	$5\times10^{-5}M$
	(% BOUND $\pm$ S.D.)	
0	85.45 ± 2.00	81.13 ± 2.42
$5\times10^{-6}M$	66.04 ± 1.64	71.68 ± 3.86
$5\times10^{-5}M$	67.72 ± 4.90	60.58 ± 2.75

TABLE X

LEUCOVORIN - 5-METHYL THF

PROTEIN-BINDING INTERACTIONS

5-METHF THF CONCENTRATION	LEU CONCENTRATION	
	5×10^{-6} M	5×10^{-5} M
	(% BOUND $\pm$ S.D.)	
0	72.49 $\pm$ 2.62	73.83 $\pm$ 1.43
5×10^{-6} M	42.53 $\pm$ 4.19	64.74 $\pm$ 4.46
5×10^{-5} M	31.36 $\pm$ 4.94	50.75 $\pm$ 1.81

7. LEU and METHF MTX Binding Interactions

Leucovorin is clinically administered to patients following the infusion of MTX. Current protocols at Roger Williams General Hospital, require that the LEU administered result in a plasma concentration ten times higher than the corresponding MTX concentration. To determine the effect of MTX on the binding of LEU the two were ultrafiltered simultaneously using two combinations of drug. In each case LEU was present in ten fold excess; first at $1x10^{-6}$ M in the presence of $1x10^{-7}$ M, then at $1x10^{-5}$ M with $1x10^{-6}$ M MTX added. Table XI shows the effect of MTX on the binding of LEU. No significant difference (t test, $p<0.05$) was observed in LEU binding with MTX.

Since METHF is the predominant circulating folate the interaction with MTX was also determined. The same scheme of concentrations as described above was used. The effect of methotrexate on the binding of METHF is shown in Table XII. The degree of METHF binding at $1x10^{-5}$ M did not change significantly (t test, $p<0.05$) in combination with MTX.

Methotrexate had a significant effect (t test, $p<0.05$) on the binding of METHF with concentrations of $1x10^{-7}$M and $1x10^{-6}$M respectively. However, rather than displacing METHF from a binding site, thereby decreasing the extent of binding, the binding actually increased. The increase in binding was from $84.20\% \pm 2.67\%$ to $97.19\% \pm 2.11$, which is

TABLE XI

LEUCOVORIN

PROTEIN-BINDING INTERACTIONS

WITH METHOTREXATE

MTX CONCENTRATION	LEU CONCENTRATION	
	1×10^{-6}M	1×10^{-5}M
	(% BOUND $\pm$ S.D.)	
0	68.44 ± 0.72	71.94 ± 3.54
1×10^{-6}M	----------	66.85 ± 1.50
1×10^{-7}M	69.23 ± 3.95	-----------

TABLE XII

METHF

PROTEIN-BINDING INTERACTIONS

WITH METHOTREXATE

MTX CONCENTRATION	METHF CONCENTRATION	
	$1x10^{-6}M$	$1x10^{-5}M$
	(% BOUND $\pm$ S.D.)	
0	84.20 ± 2.68	81.00 ± 1.71
$1x10^{-6}M$	----------	78.73 ± 3.89
$1x10^{-7}M$	$97.19 \pm 2.11*$	----------

*Significant at 0.05 Level

highly significant. An increase in the percent bound under these conditions is unexpected and cannot be readily explained. The increase may be the result of assay error since the determinations of free concentrations of METHF with total concentrations of $5x10^{-7}M$ requires measuring between $1x10^{-7}M$ and $5x10^{-8}M$, the lower limit of detection.

These results indicate that, since no differences in binding were found, MTX does not share a binding site with LEU. The same conclusion can be reached for METHF even though the degree of binding was signficantly increased in the presence of MTX at one concentration. If a binding site were shared, the degree of binding might logically be expected to decrease, not increase as was the case. Moreover, the binding of METHF did not change with MTX at higher concentration. It is therefore unlikely that MTX and METHF share a common binding site.

E. LEU Pharmacokinetics

1. Normal Subjects

The disposition of intravenously administered LEU has been characterized in 6 healthy volunteers; 3 males and 3 females. Figures 30 and 31 show the serum clearance of the drug following the administration of 10 mg. to the male and female subjects respectively. In both groups the disappearance of LEU was characterized by an initial rapid distribution phase followed by a considerably slower elimination phase.
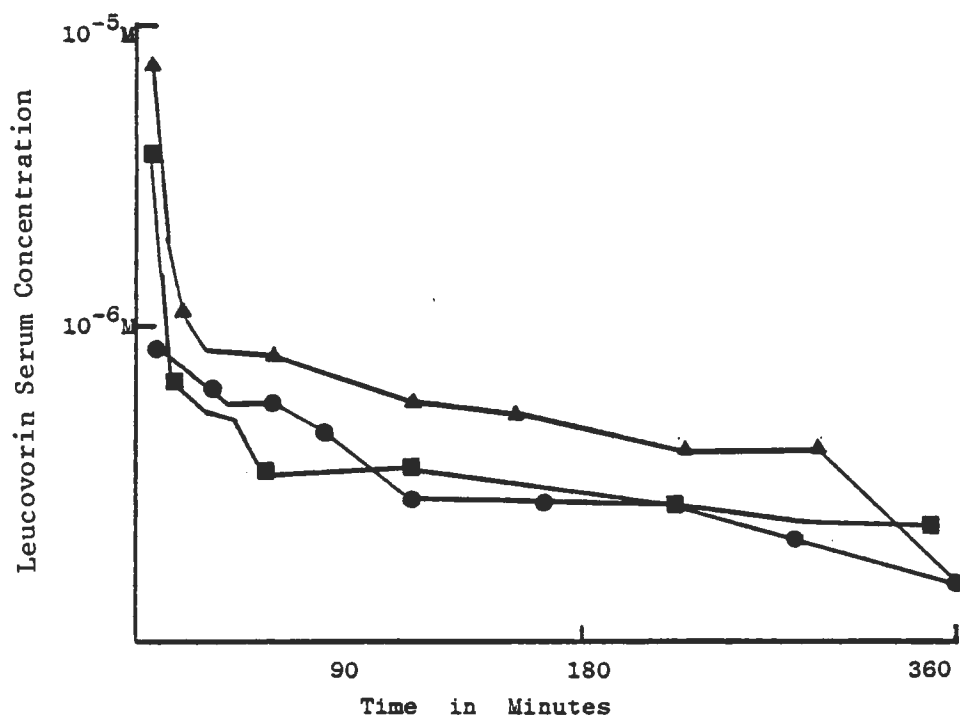
Figure 30: Leucovorin Serum Concentration as a Function of
Time; Normal Males

▲ – EM,   ● = BB,   ■ = KM
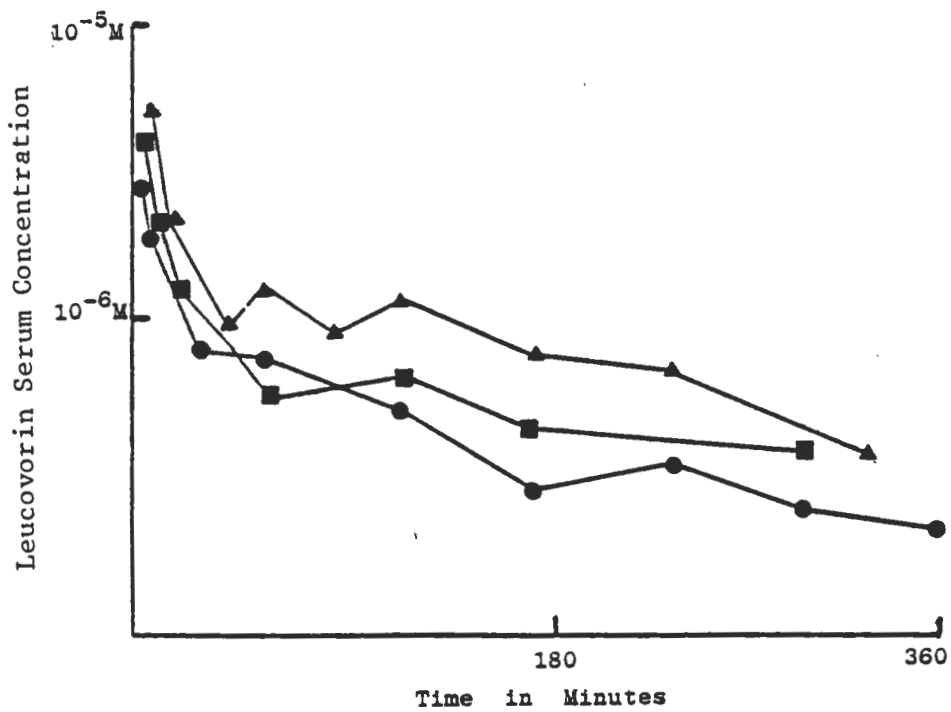
Figure 31: Leucovorin Serum Concentration as a Function of Time; Normal Females

▲ = LG,   ■ = GM,   ● = LB

LEU data for the serum concentration as a function of time was first analyzed using an ITEL AS/5 Computer system and a decision-making pharmacokinetic computer program: AUTOAN (Sedman and Wagner, 1974). This program assumes a first order elimination rate constant and examines the fit of a one, two, or three compartment open model to the data. The number of terms in the polyexponential equation is determined, an appropriate model is selected, and the microscopic rate constants, and volume of distribution are estimated. (Sedman and Wagner, 1974). The serum data for the concentration as a function of time for all 6 subjects was determined to be best described by a two compartment open model with elimination occuring solely from the central compartment (Figure 32). The biexponential equation which describes the plasma concentration as a function of time is;

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

(Equation 19)

Where A and B are pre-exponential terms and alpha and beta represent the rate constants for distribution and elimination respectively (Curry, 1974).

These values may also be expressed in terms of the micro-constants k_{12}, k_{el}, and k_{21}. These expressions as well as their derivations have been described in detail (Gibaldi and Perrier, 1975).
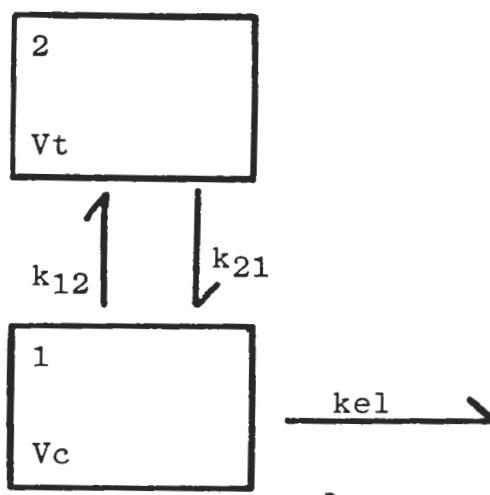
Figure 32: Two Compartment Open-Model

The initial estimates provided by AUTOAN were further refined using a second computer program, NONLIN (Metzler, Elfring, and McEwan, 1974). The pharmacokinetic model, in this case the two compartment open-model, is specified and those values of the pertinent parameters are chosen which give the best fit of the experimental data to the model. The fitting process which is employed in NONLIN is an iterative technique using non-linear least squares regression (Metzler, Elfrin, and McEwan, 1974). Four weight specifications for the concentration function were evaluated; the weighting scheme which provided the best fit of the model to the data was used to obtain the final values of the pharmacokinetic parameters. In addition, a subroutine program, DFUNC, was written to provide the necessary equations to fit the data was written and appears in Appendix IV.

The pharmacokinetic parameters describing the dispositiion of intravenously administered LEU in all six subjects are given in Table IV. Although the microconstants have relatively small standard deviations associated with them, the volumes of distribution show a great deal of variability. This can most likely be attributed to the large difference in the weights of the normal volunteers, e.g. 45 Kg to 100 Kg. Because the height of the 6 subjects also varied greatly (over a 31 cm. range) the surface areas calculated for the volunteers showed a related variability; 1.37 m^2 to 2.28 m^2.

TABLE XIII

LEUCOVORIN

PHARMACOKINETIC PARAMETERS CALCULATED

FROM NORMAL VOLUNTEERS

Parameter		All Volunteers (Mean ± S.D.)		
Kel^a	Min. - 1	1.54	±	0.88
$K12^a$	Min. - 1	0.93	±	0.29
$K21^a$	Min. - 1	1.96	±	0.50
Vcl	ml *Min. - 1	6.21	±	0.21
Vd (Cen.)	Liters	5.49	±	3.53
Vd (SS)	Liters	8.44	±	5.73
Vd (Beta)	Liters	21.12	±	8.95
Vd (Extrap)	Liters	24.10	±	9.39
Vd (Tiss)	Liters	2.95	±	2.23
Half-Life (Alpha)	Minute	8.79	±	4.44
Half-Life (Beta)	Minute	231.46	±	31.76

Mean ± SD, (n=3)

$a = x \ 10^2$

Normalizing the volumes of distribution by expressing these terms as either ml Kg⁻¹ or ml m⁻² rather than as ml greatly reduces the effects of subject variability in these parameters.

The description of a biexponential decay in LEU serum concentration as a function of time is in agreement with the report of Rothenberg et al. (1979) in which a "rapid" decline was followed by a second prolonged elimination phase which approaches a "plateau" in appearance. A similar rapid distribution phase (alpha half-life 8.79 ± 4.44 minutes) and a "slow elimination phase (beta half-life 231.46 ± 31.76 minutes) have been characterized in the present study. Rothenberg et al. (1979) have suggested that the plateau results from the selective elimination or tissue uptake of only the active form of LEU, leaving the inactive diastereoisomer in the systemic circulation for prolonged periods. The present work can neither confirm or refute such a suggestion since the assay method does not distinguish between forms of folinic acid, nor was the active diastereoisomer available for administration to the subjects. The total body clearance of the drug was calculated as 62.1 ml min⁻¹ ± 21.3 ml min⁻¹. Clearance has been calculated according to the following equation ;

$$V_{cl} = \beta * Vd_{\beta}$$

(Equation 20)

The large standard deviation evident in this parameter is most likely caused by the previously described variability in volumes of distribution.

Literature values reported for the percent of LEU excreted unchanged may be used to approximate the renal and metabolic clearance of LEU. Spray and Witts (1953) reported that an average of 15% of the administered drug was excreted in the urine in two hours. Rothenberg et al. (1979) calculated a value of approximately 18% for the amount excreted unchanged. An average value obtained from these two reports, 16.5, was used to estimate a renal and a metabolic clearance for LEU. The total body clearance for LEU (Vcl) was calculated to be an average of 62 ml min^{-1}. The renal excretion can be calculated according to Equation 21;

$$Cl_{(renal)} = GFR + AS - TR$$

(Equation 21)

The renal clearance of LEU was calculated to be 10.23 ml min^{-1}. Subtracting this value from Vcl yields the metabolic clearance: 51.77 ml min^{-1}.

The 6 normal subjects were assumed to have a normal glomerular filtration rate of 120 ml min^{-1}. Only the free, non-protein bound, fraction of circulating drug is subject to glomerular filtration.

LEU. The contribution of glomerular filtration for LEU, assuming the fraction free to be 30% was calculated as 36 ml min⁻¹. Since LEU's calculated renal clearance, 10.23 ml min⁻¹, is less than the calculated maximum glomerular filtration rate, there is assumed to be tubular reabsorption of LEU. The extent of reabsorption can be obtained from the difference between glomerular filtration and renal clearance. The net tubular reabsorption rate for LEU was calculated to be approximately 26 ml min⁻¹.

A comparison of the pharmacokinetic parameters derived from the male and female volunteers is presented in Table 3. No statistical differences ($p < 0.05$) were observed in any of the pharmacokinetic parameters calculated.

2. Cancer Patients

The disposition of LEU was followed in 4 patients undergoing High-Dose MTX therapy at Roger Williams General Hospital. One patient, L.M., received 2 courses of treatment approximately 1 month apart and LEU disposition was characterized following both courses. Three patients received the drug intravenously, the fourth was given LEU by intramuscular injection.

In 3 of the data sets the diappearance of LEU following intravenous administration was characterized by an initial rapid distributive phase followed by a slower elimination phase. Figures 33 and 34 show the clearance of the drug in patient F.F and L.M. The biexponential decay

was in agreement with the pattern observed in normal subjects. The serum concentration-time data for these patients was analyzed first using AUTOAN then NONLIN to provide the final values of all parameters. Table XV shows the values of the pharmacokinetic parameters derived from the cancer patients and normal male subjects. No statistical differences (p <0.05) were found between parameter estimates in the normal subjects versus cancer patients.

Lacking pharmacokinetic parameters for LEU, the rescue protocol at Roger Williams General Hospital was developed assuming that its disposition is similar to that of MTX. Leucovorin rescue has been dosed to provide an initial plasma concentration ten-fold higher than the projected MTX concentration in the patient 30 hours after the termination of the MTX infusion. Table XVI is a comparison of the expected LEU serum concentration and the calculated initial serum concentration determined by electrochemical assay. The calculated serum concentration averaged 6.67 times (range 3.5 to 11.8) greater than the expected concentration. Although the excess LEU will not result in toxicity, the net effect is to reduce the effectiveness of MTX therapy. The disparity between observed and expected serum concentrations may be largely attributed to the differences in volume of distribution between LEU and MTX. Table XVII shows a comparison of the half-lives and volumes

TABLE XIV

A COMPARISON OF

LEUCOVORIN

PHARMACOKINETIC PARAMETERS CALCULATED

FROM FEMALES VS. MALES*

Parameter		Males (Mean ± S.D.)			Females (Mean ± S.D.)		
Kel[a]	Min.-1	1.14	±	0.43	1.93	±	0.11
K12[a]	Min.-1	1.12	±	0.23	7.33	±	0.20
K21[a]	Min.-1	2.25	±	0.49	1.68	±	0.39
Vd (Cen.)	Liter	7.41	±	3.48	3.58	±	2.85
Vd (SS)	Liter	11.31	±	5.62	5.51	±	5.08
Vd (Beta)	Liter	26.40	±	9.24	15.97	±	5.77
Vd (Ext.)	Liter	29.23	±	9.78	18.97	±	6.76
Vcl[a]	Ml/Min.	7.49	±	1.94	4.94	±	1.64
Vd (Tiss)	Liter	3.90	±	2.16	2.00	±	2.26
t1/2	Min.	8.66	±	2.98	8.93	±	6.36
t1/2	Min.	240.48	±	44.03	222.43	±	18.41

*No Statistical Differences (p<0.05)

a = x 10^2

Figure 33: Leucovorin Serum Concentration as a
Function of Time; Patient F.F.
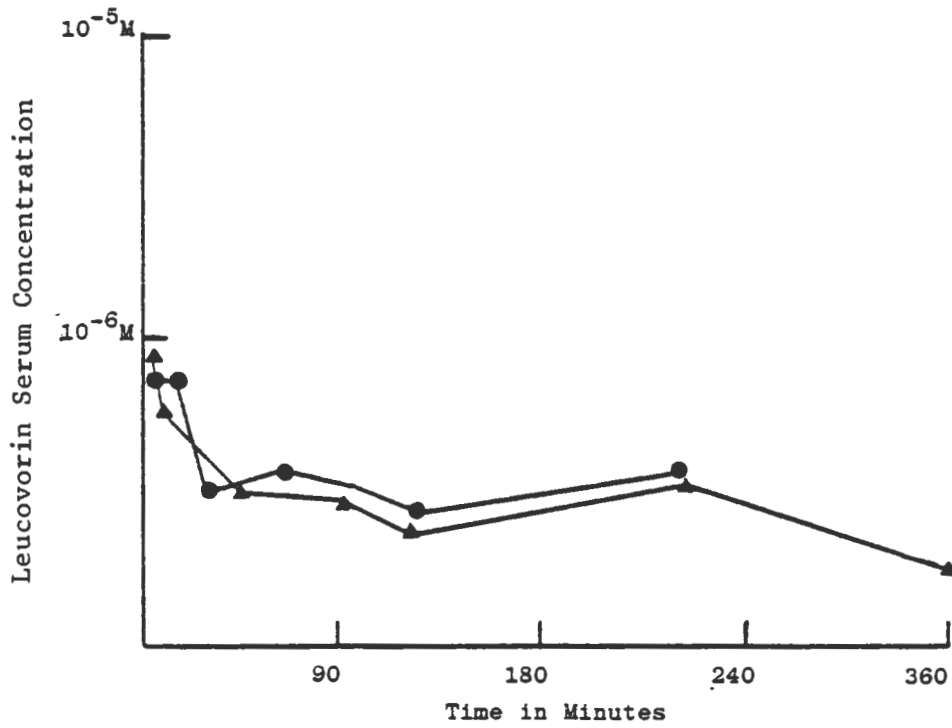
Figure 34: Leucovorin Serum Concentration as a Function of Time; Patient L.M.

Treatment Period 1

Treatment Period 2

TABLE XV

A COMPARISON OF LEUCOVORIN PHARMACOKINETIC

PARAMETERS DERIVED FROM NORMAL SUBJECTS

AND CANCER PATIENTS

Parameter		Normal Volunteers* (Mean ± S.D.)			Cancer Patients* (Mean ± S.D.)		
Kel[a]	Min.-1	1.14	±	0.43	1.20	±	0.69
K12[a]	Min.-1	1.12	±	0.23	1.16	±	0.21
K21[a]	Min.-1	2.25	±	0.49	2.90	±	0.60
Vd (Cen.)	Liters	7.41	±	3.48	4.06	±	0.73
Vd (SS)	Liters	11.31	±	5.62	5.69	±	0.96
Vd (Beta)	Liters	26.40	±	9.24	17.51	±	2.26
Vd (Ext.)	Liters	29.23	±	9.78	19.07	±	2.68
Vd (Tiss.)	Liters	3.90	±	2.16	1.63	±	0.24
Clearance	Ml/Min	74.9	±	19.4	46.4	±	20.4
$t\frac{1}{2}$ (Alpha)	Min	8.66	±	2.98	5.62	±	0.59
$t\frac{1}{2}$ (Beta)	Min	240.48	±	44.03	2.68	±	131.43

* n = 3

a = x 10^2

TABLE XVI

COMPARISON OF EXPECTED INITIAL LEUCOVORIN SERUM

CONCENTRATIONS TO CALCULATED LEVELS

Patient	Expected Concentration (Moles/Liter)	Calculated Concentration (Moles/Liter)
F.F.	1.50×10^{-7}	1.77×10^{-6}
L.M. -1	3.50×10^{-7}	1.65×10^{-6}
L.M. -2	4.20×10^{-7}	1.47×10^{-6}

TABLE XVII

COMPARISON OF PHARMACOKINETIC PARAMETERS

FOR LEUCOVORIN AND METHOTREXATE

Parameter	LEU (Mean ± S.D.)			MTX (Mean ± S.D.)		
Half Life (Alpha) (Min.)	8.79	±	4.44	44 min. ±		6.6
Half Life (Beta) (Min.)	231.46	±	31.76	120 min. -		240
Half Life (Gamma) (Min.)	------------			624 min. -		1620
Vd (Central) (Liters)	5.49	±	3.53	13.09	±	1.09
Vd (SS) (Liters)	8.44	±	5.73	73.3	±	15.6
Binding to HSA	70%			60 - 87%, 45%		

of distribution reported for MTX. and the values obtained for LEU in the current study. It is evident from these data that MTX pharmacokinetic parameters differ greatly from those of LEU and therefore cannot be used to correctly predict its disposition. Two patients from whom serum samples were obtained showed patterns of LEU disposition distinctly different from other subjects. Patient P.F. received LEU, 1.1 mg m^{-2}, intravenously 36 hours after the end of MTX infusion. A plot of the serum LEU concentration as a function of time is shown in Figure 35. Rather than the biphasic disposition evident in the other cancer patients and all normal subjects, an essentially constant level of LEU was detected in the patients serum. During the period of sample collection, hour 36 to 42 post infusion, a change in the elimination of MTX in this patient was observed. The half-life (beta) had increased from the normal value of 2 to 4 hours to approximately 22 hours indicating a significant decrease in the rate of MTX clearance. No indication of changes in P.F.'s renal or hepatic function were observed during the change in MTX clearance and the event has been unexplained. It is possible however, that the cause of decreased MTX clearance observed in this patient may also be responsible for the change in the disposition of LEU which was observed in P.F.

Patient F.Y. received 12 mg. of LEU intramuscularly 48 hours following the end of MTX infusion. This was the fourth dose administered to the patient. Figure 36 is the plot of the LEU serum concentration as a function of time for F.Y. The serum concentration remained essentially constant over the 2 hour sampling period. This may be explained by the release and or absorption characteristics of the drug from the intramuscular injection site. More data would be required to be able to characterize the intramuscular route of administration as providing a slow release rate of LEU and acting as a slow-infusion.

3. Comparison of Leucovorin Pharmacokinetics derived from Microbiologic Versus Electrochemical Assay Methods

Spray and Witts (1953) described the utilization of intravenously administered LEU in normal subjects. LEU was assayed using the microbiologic disc method of Sauberlich and Baumann (1948). Recently Rothenberg et al. (1979) published a radiochemical assay for LEU in serum and urine. The disposition of LEU characterized by the two methods are very much different. The early work describes a rapid decline in serum concentration whereas Rothenberg et al. (1979) found a second slow elimination phase after the initial distributive phase. A comparison of pharmacokinetic parameters derived from the two methods was
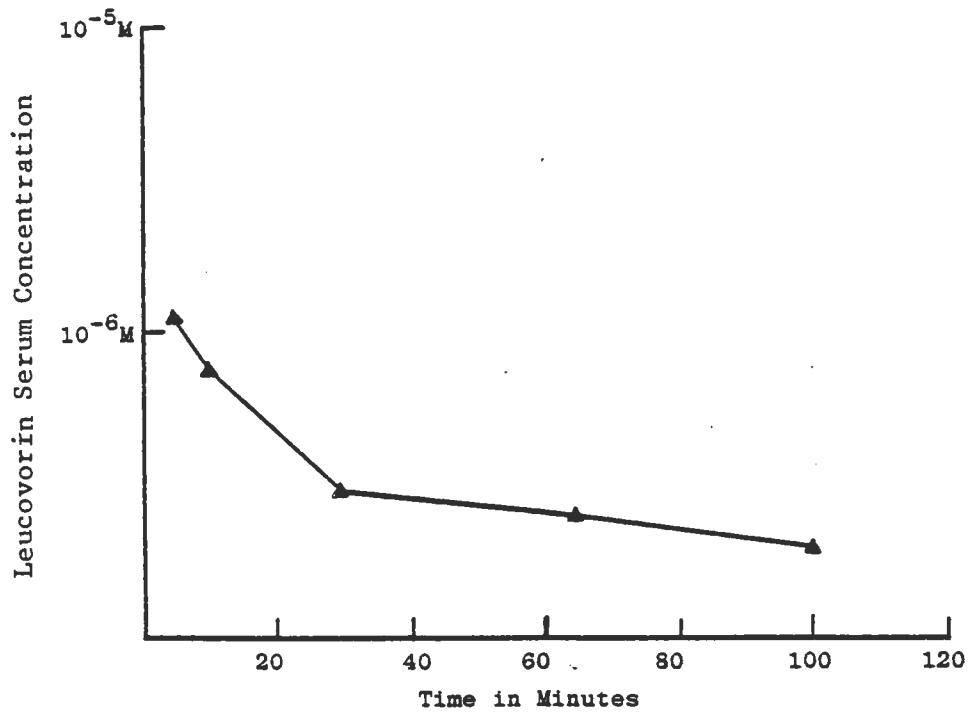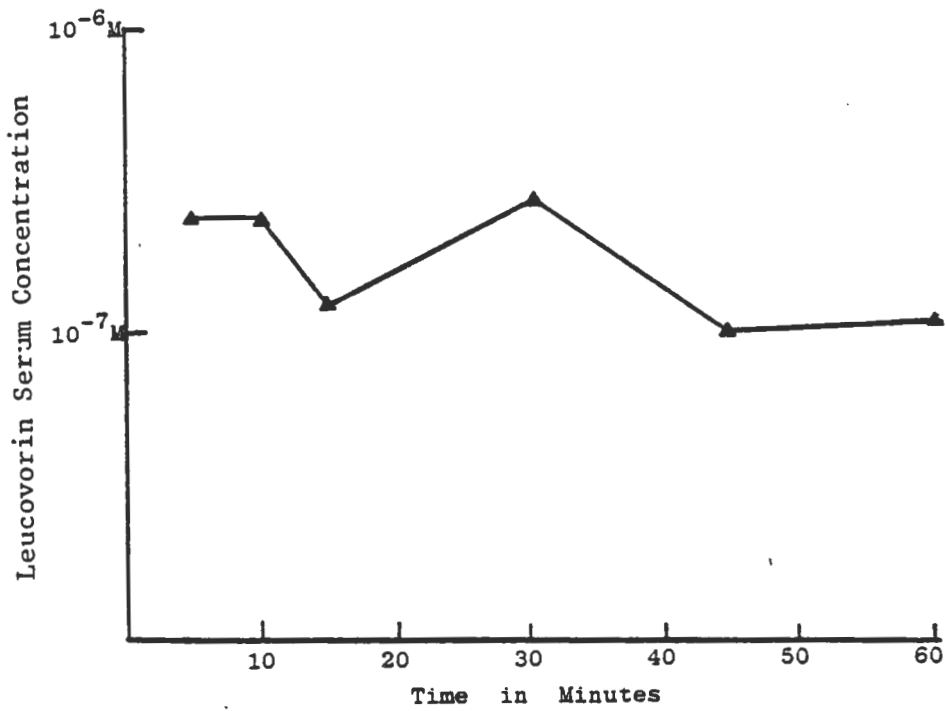
Figure 35:   Leucovorin Serum Concentration as a Function of
Time; Patient P.F.
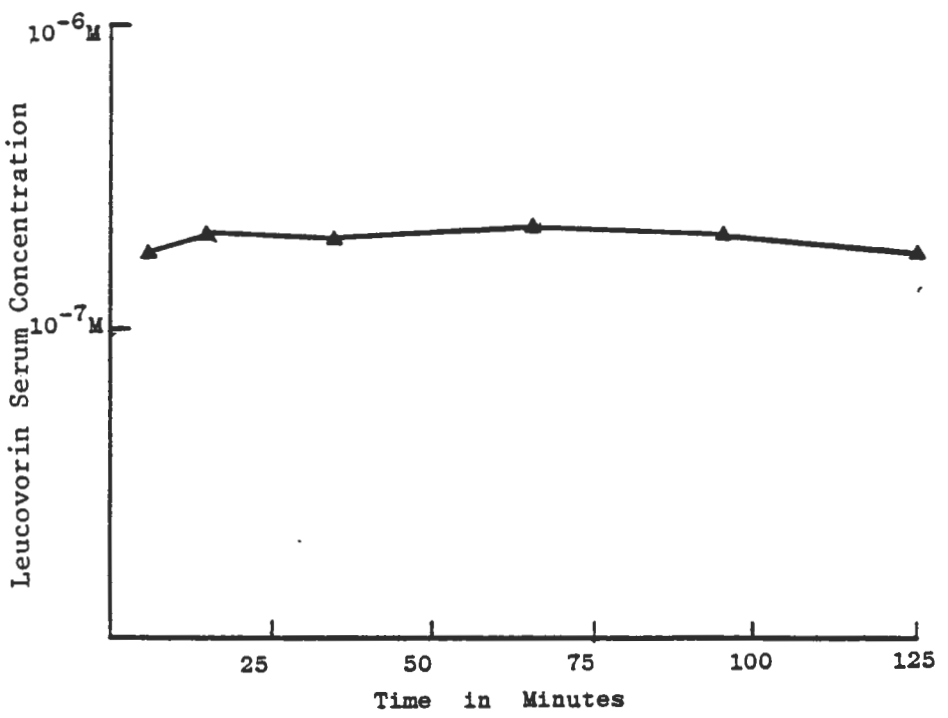
Figure 36: Leucovorin Serum Concentration as a Function
of Time; Patient F.Y.

performed using the plasma concentration-time data published by Spray and Witts (1953).

The data obtained from three normal male subjects given 1 mg. of LEU intravenously (Spray and Witts, 1953), were first analyzed using AUTOAN to yield initial estimates of the microconstants. The data appears to be best described by a two compartment open model as was the case in the current work. These estimates were then used to obtain final values for the pharmacokinetic parameters using NONLIN as previously described.

Table XVII shows the parameters derived from each assay method in 3 normal male subjects. Statistical differences (p <0.05) were found in several of the parameters; kel, k12, alpha, beta, Vd (extrap.), clearance and half-life (beta). Figure 37 is a computer generated graph of serum concentration as a function of time using the parameters derived from the LEU serum analyses. The y-axis intercepts are different because the studies used different doses in the subjects. Evident in Figure 37 is the apparently faster rate of elimination described by the microbiologic method. The half-life (beta) is 32.03 ± 3.82 min versus 231.46 ± 31.76 min when measured by the electrochemical method. Rothenberg et al. (1979) attributes the difference in elimination rate evident between the microbiologic and radiochemical methods to the inability of the latter to distinguish between the active and inactive diastereoisomers of LEU. They have further

speculated that the sustained elevated serum LEU concentration is caused by a preferrential excretion of the active isomer. Conclusions regarding the excretion of only the active form of LEU are not possible from the current work. However, these data are in agreement with Rothenberg et al. (1979) in describing a prolonged elimination phase.

TABLE XVIII

A COMPARISON OF

PHARMACOKINETIC PARAMETERS CALCULATED

FROM MICROBIOLOGIC ASSAY

VS. ECD METHOD

Parameter		Microbiologic (Mean $\pm$ S.D.)			Electrochemical (Mean $\pm$ S.D.)		
Kel[*a]	Min.-1	6.15	$\pm$	4.60	1.14	$\pm$	0.43
K12[*a]	Min.-1	1.79	$\pm$	0.37	1.12	$\pm$	0.23
K21[*a]	Min.-1	3.20	$\pm$	0.28	2.25	$\pm$	0.49
Vcl	Ml *Min.-1	56.10	$\pm$	12.70	7.49	$\pm$	1.94
Vd (Cen.)	Liters	9.14	$\pm$	2.07	7.41	$\pm$	3.48
Vd (SS)	Liters	14.20	$\pm$	2.12	11.31	$\pm$	5.62
Vd (Beta)	Liters	25.48	$\pm$	3.56	26.40	$\pm$	9.24
Vd (Extrap)	Liters	62.18	$\pm$	10.18	29.23	$\pm$	9.78
Vd (Tiss)	Liters						
Half-Life (Alpha)	Min.	7.81	$\pm$	0.68	8.66	$\pm$	2.98
*Half-Life (Beta)	Min.	32.08	$\pm$	3.82	240.48	$\pm$	44.03

*Statistically Different ($p < 0.05$)

$a = \times 10^2$
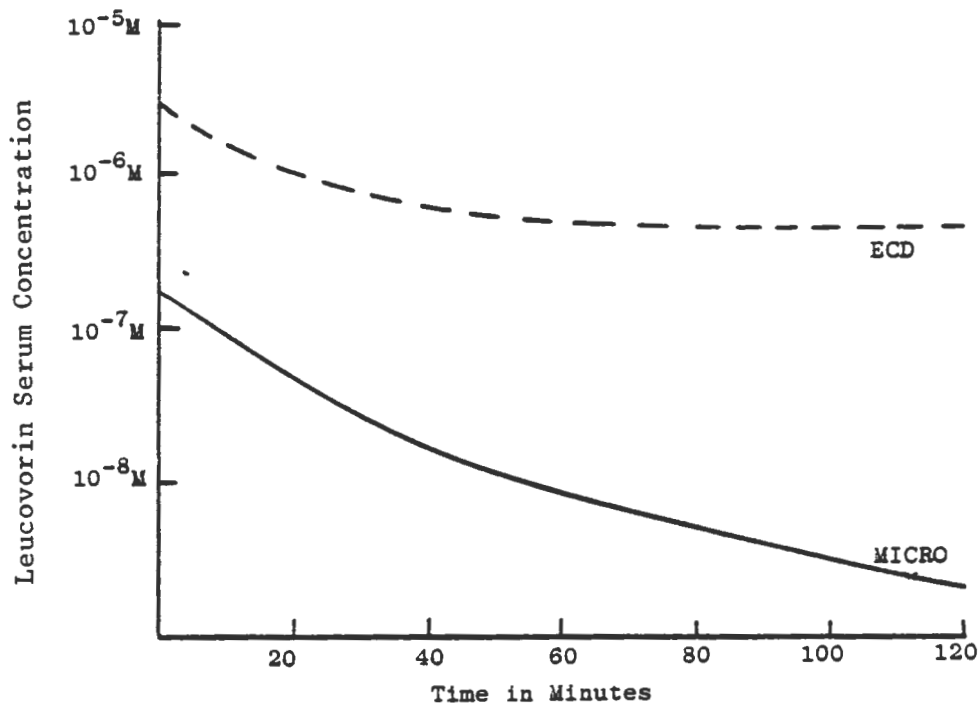
Figure 37: Leucovorin Serum Concentration as a Function of Time. A Comparison Using Pharmacokinetic Parameters Derived from Two Assay Methods. ECD = Electrochemical; Micro = Microbiologic.

XI. CONCLUSIONS

1. High pressure liquid chromatography was coupled with electrochemical detection and a sensitive, selective, and reproducible assay for LEU and METHF was developed.

2. The electroanalytical detection of reduced folates relies on the oxidation of the electroactive species at a surface electrode. The rate of oxidation, and therefore the generated current, can be affected by the electrode material, the buffer strength of the supporting electrolyte, and the strength of the applied potential. Oil-based, wax-based, and solid electrodes were investigated; solid glassy carbon electrodes were found to provide the optimum balance of durability and sensitivity. Ammonium phosphate, dibasic, was determined to be a suitable supporting electrolyte over a wide pH range, with which low background current and adequate sensitivity was achieved. Raising the applied potential increased, up to a limit, the background current and the current produced via oxidation of electroactive species. An applied potential of 0.8 volts produced adequate sensitivity for the reduced folates and minimized the oxidation of interfering compounds from serum samples. METHF was more easily oxidized than LEU, with a limiting current of 0.3 volts

compared to 0.8 volts for LEU. The lower limit of detection for these folates was approximately 12 ng.

3.   Reverse-phase paired-ion chromatography was used to effect the separation of LEU and METHF in aqueous systems and the separation of LEU from the extracted components of human serum. However, this method was not of adequate efficiency to separate METHF from all components of serum. The restraints upon the chromatographic conditions imposed by electrochemical detection severely limited the efficiency of octadecyl silane stationary phases (C-18 columns). In order to effect separation of LEU from other plasma constituents it was necessary to use the recently developed RCSS methodology. This technique was evaluated in considerable detail and found to offer several significant advantages over conventional reverse-phase columns.

4.   A method for the extraction and essentially complete recovery of LEU from human serum has been developed. The applicability of this technique to other forms of reduced folates would depend upon their aqueous and organic solubilites, ability to form a paired-ion complex, and stability during evaporation and storage.

5.   The binding of LEU and METHF to human serum albumin was determined. Binding was not saturated over the drug

concentration range investigated. The presence of both LEU and METHF in the albumin solution significantly reduced the degree of binding for both drugs. Since other forms of folates are found to varying degrees in human blood, it is possible that _in_ _vivo_ binding of the reduced folates is substantially less than was determined from _in_ _vitro_ experiments.

The addition of MTX to albumin solutions containing LEU or METHF had no significant effect on the degree of binding of the reduced folates. Thus, it is concluded that MTX and the two reduced folates studied probably do not share a common binding site. The apparent absence of a protein binding interaction is important clinically. From these data it may be concluded that the administration of LEU following high-dose MTX therapy would not likely alter the equilibrium between free and bound plasma MTX amounts or cause a redistribution of intracellular or extracellular MTX. No conclusions, however, can be drawn regarding the possible interaction between LEU, METHF, and MTX for proposed active transport mechanisms into cells.

6. The pharmacokinetics of LEU were characterized in 6 normal subjects and in 5 patients undergoing high-dose MTX therapy. No significant differences in pharmacokinetic parameters between the two groups were observed. LEU dosing protocols aim for concentrations that are ten times higher than are necessary for effective rescue. The current work

suggests that such protocols for LEU dosing, based on MTX pharmacokinetics, result in plasma or serum LEU concentration in excess of even these guidlines. Although high serum LEU concentration pose no threat of toxicity, and can effectively reduce MTX related toxicity, the effectiveness of the high-dose MTX therapy may be reduced.

It may be concluded that LEU concentrations achieved in cancer patients are higher than necessary for effective rescue and, most importantly, muy decrease the effectiveness of MTX. Based on the pharmacokinetic parameters calculated from cancer patients, a reduction of 50% in LEU doses may be possible and would be expected to: 1. improve the efficacy of MTX therapy and; 2. maintain the effectiveness of LEU rescue regimens.

Future Work

Several possibilites for further investigation have resulted from this work:

1. The electrochemical requirement for relatively high ionic strength buffers may substantially reduce the efficiency of some high pressure liquid chromatographic systems. In the current work calculated column efficiencies were reduced by approximately 80% from the claimed column efficiencies.

The use of post-column addition of supporting

electrolyte may represent a solution to this problem. In this manner, chromatographic conditions may be optimized with fewer concessions to electrochemistry than are currently possible. Such an approach might be expected to produce greater resolution and efficiencies in HPLC and most likely lower the limits of sensitivity.

2. The renal clearance of LEU has received little attention. Furthermore, published reports have not adequately performed urine sampling with regard to proper pharmacokinetic analysis. Therefore, reliable estimates of the total amount of LEU excreted in urine are not available. The current assay method could be applied to an adequately designed pharmacokinetic study, resulting in the characterization of the renal and metabolic clearances of LEU.

3. The extraction procedure described in the current work was not suitable for the analysis of METHF. METHF can be quantitatively determined at an oxidation potential of 0.3 Volts, rather than the 0.8 Volts necessary for the oxidation of LEU. By decreasing the applied potential, the oxidation of serum constituents is expected to be reduced considerably. Under this condition it may be possible to develop an extraction process for METHF that would allow for the determination of its pharmacokinetics in humans.

4.   There is considerable interest in qualitative and quantitative analysis of reduced folates (monoglutamates) and various polyglutamates within cell. The concentrations of these compounds is below the limit of sensitivity achieved for the reduced folates. Modifications in the electrochemical cell, including increased electrolyte cell surface, repositioning of the auxiliary electrode, and decreasing the diffusion distance within the cell may be expected to provide greater levels of sensitivity. Coupled with the improved chromatographic techniques suggested above, this approach may lead to the development of a system suitable for the detection of intracellular folates and polyglutamates.

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INSTITUTIONAL REVIEW BOARD

REPORT OF ACTION

TITLE OF PROPOSAL: "Pharmacokinetics of Leucovorin in Normal Subjects"

PRINCIPAL INVESTICATOR: D.S. Greene

This is to certify that the application identified above has been reviewed by the Board approved to review proposals for research and other activities involving human subjects.

DATE OF REVIEW: February 19, 1980

COMMITTEE ACTION: Approved.

COMMENTS.

_____
I.R.B. Chair

Cc: P.I.
    Dept. Chair
    Coordinator of Research

ROGER WILLIAMS
GENERAL HOSPITAL

825 CHALKSTONE AVENUE
PROVIDENCE, RHODE ISLAND 02908

Nursing Instructions for High Dose Methotrexate-Leucovorin Rescue Protocol

MTX Dose— Initial course 1000mg/m^2 intravenously over 18 hours with 10% of the total dose by rapid intravenous infusion over 30 minutes. Subsequent courses MTX dose to be modified in order to achieve a plasma level of 1×10^{-4} M during the MTX infusion.

Hydration— Intravenous infusion of 3000ml/m^2/24hrs. with 88meq HCO_3/m^2/24hrs. (2 amps) to be started 12 hours prior to the MTX infusion and to be continued until completion of leucovorin rescue (hour 42).

Urine pH— MTX infusion should not be started unless the patient's urinary pH$\geq$7.0. Urinary pH should be monitored periodically for 24hrs. following the start of MTX. The houseofficer should be notified immediately if the urinary pH falls below 7.0.

Plasma MTX— Blood (2ml in 200units of heparin) should be drawn 9, 18, 24, 30, 36, 42, and 48hrs. following the start of MTX. Bloods should be clearly labelled with the patient's name and hour drawn and should be stored in the refrigerator on West-4.

Leucovorin — Leucovorin rescue will be given intravenously at 30, 36, and 42hrs. after the start of the MTX infusion. Leucovorin dose is to be calculated on the basis of the 9, 18, and 24hr. plasma MTX levels. It is therefore crucial that these levels be drawn on time and that the laboratory be notified immediately after the 24hr. MTX level is drawn (X2087 JoAnn Aspry or Joyce Blaisdell). The Oncology fellow will notify the West-4 Unit once the leucovorin dose has been calculated. If no such notification has been received by hour 30, contact Dr. Ron Stoller immediately.

Bloods— Pretreatment: CBC, platelet count, differential, BUN, serum creatinine, creatinine clearance, bilirubin, alkaline phosphatase, SGOT, and urinalysis.
Follow-up: Daily CBC, platelet count, BUN, serum creatinine, alkaline phosphatase, SGOT x 3days.

If there are any questions please contact Dr. Ron Stoller at X2055 or 2060.

CONSENT FORM

THE FULL NATURE AND EXTENT OF YOUR DISEASE HAS BEEN EXPLAINED TO YOU.

1. You are invited to participate in the following study: High-Dose
    Methotrexate with Leucovorin Rescue: A Clinical and Pharmacological
    Study.

        You will receive the combination of high dose methotrexate and
        leucovorin rescue.

2. If you decide to participate, the procedures to be followed are
    explained below:

        These drugs will be administered by vein. It is understood
        that the therapy will be discontinued if severe toxicity
        develops or if the therapy is found to be ineffective. Also,
        periodic tests will be performed to measure the effectiveness
        of this therapy.

3. Those procedures which are investigational have been identified as:

        The investigational portion of this study involves the measure-
        ment of the effectiveness of these drugs and your tolerance to
        them.

4. As with any investigative procedure or medication, previously
    unreported or unforeseen complications may occur rarely. The
    following complications or risks have been reported, or are known,
    or may occur:

        This combination of drugs has side effects which may include
        any, all, or none of the following: nausea, vomiting,
        diarrhea, a sore mouth and throat with or without sores similar
        to canker sores, lowering of the white blood count and/or
        red blood count and/or platelet count, increased risk of
        infection and possible kidney damage.

5. The benefits which can be expected have been described as:

        It is possible that your disease may be controlled by a
        decrease in the growth of malignant tissue in an effective
        and safe manner. It is not possible to predict whether or
        not any personal benefit will result from the use of this
        treatment program.

6. The alternate procedures, if any, which would be possibly advantageous to you have been described as follows:

> Other treatment programs which may include the drugs in this program or other combination of drugs similar to those in this program are available, but there is no clear evidence that other combinations would provide an increased chance of controlling your disease and the side effects would be similar to the drugs proposed in this study.

7. Feel free to ask any questions you have about the study and we will be happy to answer them. If you have any additional questions later, Dr._____ may be contacted through the nurse if you are hospitalized or by calling 456-2100 if you are an out-patient.

8. Any information that is obtained in connection with this study and that could identify you will remain confidential and will be disclosed only with your permission. If you give us your permission by signing this document, we plan to disclose data about you, which does not identify you by name, for medical and other scientific purposes, including publications.

9. You are making a decision whether or not to participate in this study. Your decision whether or not to participate will not prejudice your future relationship with the Roger Williams General Hospital. If you decide to participate, you are free to withdraw your consent and to discontinue your participation at anytime without prejudice.

10. Please give us an indication of your having read this document by placing your initials next to each numbered item described above.

11. Your signature on this document indicates that you have decided to participate having read the information provided above.

PATIENT:_____ DATE:_____ TIME:_____

SIGNER OTHER THAN PATIENT:_____ RELATIONSHIP:_____

PERSON EXPLAINING THE STUDY:_____ DATE:_____ TIME:_____

PRINCIPAL INVESTIGATOR:_____

WITNESS:_____ DATE:_____ TIME:_____

Roger Williams
General
Hospital

February 8, 1980

Douglas S. Greene, Ph.D.
University of Rhode Island
Department of Pharmacy
Kingston, Rhode Island 02881

Dear Dr. Greene:

As Secretary of the Ethics and Clinical Investigations Committee,
I am pleased to advise you that your Progress Report of your Grant
Application, Roger Williams General Hospital #80-248-46 titled
"Pharmacology of Leucovorin in Cancer Chemotherapy" was reviewed and
approved by the Ethics and Clinical Investigations Committee at their
meeting on February 7, 1980.

Under Federal and Committee Regulations, this Grant will automatically
be submitted for review in February of 1981. Prior to the next review, it
will be necessary for you to submit a summary regarding the activities of
this Grant.

Cordially,

Nancy P. Madeira, Secretary
Ethics & Clinical Investigations Committee

NPM/am

cc/Dr. F. Cummings
   Mr. O. Bisbano

INFORMED CONSENT FORM

Leucovorin is similar to folic acid, an essential vitamin normally contained in the diet. It is a drug normally used with methotrexate in cancer chemotherapy. Extemely large amounts have been administered to normal subjects with no ill or toxic effects.

The objective of this research is to study the rate leucovorin is removed from your body and to attempt to determine the amounts of other compounds produced by your body in your blood as a function of time.

Subjects volunteering to participate in this project will be required to abstain from all drugs and alcohol for a period of 48 hours prior to each test. In addition, all volunteers will be required to fast for twelve hours before starting the test and for at least four hours after the test has begun. The drug, Calcium Leucovorin, will be prepared using commercially available intravenous preparations. A low dose (10 mg) of leucovorin will be administered to you directly into your arm vein by a physician using a hypodermic syringe. A catheter (i.e. a small tube) will be placed in the vein of your arm for the collection of blood. Ten milliliters (about two teaspoonfuls) will be collected before you receive the dose of leucovorin and thirteen ten milliliter blood samples will be collected at fifteen minutes, thirty minutes, forty-five minutes, one hour, one and one half hours, two hours, two and one half hours, three hours, four hours, five hours, six hours, seven hours and eight hours after drug administration. All blood samples will be drawn by a physician or appropriately trained personnel.

Initial

Leucovorin has been reported to be completely free of toxic reactions except for a single report of an allergic reaction to the drug with no lasting ill effects. At the dose of leucovorin you will receive, the risk of any side effect is minimal. Risks associated with the use of a catheter for the collection of blood samples include: infection, hematoma (i.e. soreness and/or "black and blue" mark) or bleeding.

Medical care for problems resulting from this study will be available at Roger Williams General Hospital for emergencies. You will not be compensated for injuries resulting from this study.

You are free to withdraw from participation in this study at any time during the investigation. You will not be identified in any publication resulting from this work.

Feel free to ask any questions you have about the study and we will be happy to answer them. If you have any additional questions later, Dr. _____ may be contacted through the investigators or by calling 456-2060.

I, _____, having carefully read and understood the above and having been given the opportunity of asking questions regarding the above project now give my informed consent to participation in the study of Leucovorin Pharmacokinetics in Normal Subjects, University of Rhode Island.

Date_____   Volunteer: _____

Person explaining the study: _____

Principal Investigator: _____

Witness: _____   Date: _____

All those being considered for involvement in this study will be healthy

~~male~~   volunteers between the ages of twenty and fifty with no known

hypersensivity to folic acid or its derivatives. Any medical abnormality,

which, in the opinion of the medical consultant or investigators, is

likely to complicate the study or add further risk to the subject will

result in the subject being excluded from the study. All subjects will

be carefully interviewed and pertinent medical information gathered.

All participants will be provided with the opportunity of reading and

having carefully explained the Informed Consent Form. In addition,

participants will be provided the opportunity for further questioning

of the investigators. Any subject may withdraw from the study at any

time during the investigation. Medical care will be provided at

Roger Williams General Hospital for medical emergencies resulting

from this study. Subjects will receive no compensation for injuries

received during this study.

Participants will be required to abstain from all drugs and alcohol

for 48 hours prior to the test period. In addition, participants will be

required to fast twelve hours before drug administration and for at

least four hours after drug administration.

All drug administration and blood sample collection will be

under the direction of a physician at Roger Williams General Hospital

in Providence, RI.

Calcium leucovorin will be administered intravenously using

commercially available preparations at a dose of 50 mg. Blood samples

will be collected immediately before drug administration and at 0.25,

0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 8 hours after drug

administration through an indwelling cathetor. The test will require

that ten milliliters of blood be taken at each interval for a total of 140 milliters from each volunteer for each test. The risk to the subjects from the administration of the drug is judged to be minimal, doses of over 500 mg have been administered to normal subjects with no toxic effects. Risks resulting from the collection of blood include the possibility of infection, hemotoma and scarring. The amount of blood collected in not excessive, this is less than one-third of a pint.