The Development of HPLC Methods for the Determination of Methotrexate and Doxorubicin Metabolites and their Application to Clinical Studies.

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ABBREVIATIONS

ADR	Doxorubicin
ADRol	Doxorubicinol
ADRone	Doxorubicin Aglycone
amu	Atomic Mass Unit
AUC	Area Under Curve
Cl	Clearance
Cl_m	Metabolic Clearance
Cl_R	Renal Clearance
Cr_{Cl}	Creatinine Clearance
CV	Coefficient Variation
DAMPA	4-Amino N^{10}-methylpteroic Acid
DAUN	Daunorubicin
DHFR	Dihydrofolic Acid Reductase
F	Absorption Factor
f_e	Fraction of Drug Excreted Unchanged
h	Reduced Height Plate
HPLC	High Performance Liquid Chromatography
IM	Intramuscular
IS	Internal Standard
IT	Intrathecal
IV	Intravenous
k'	Capacity Ratio
K_{el}	Serum Elimination Rate Constant
M^+	Mass Ion
MTX	Methotrexate
$MTXG_1$	Methotrexate Diglutamate
$MTXG_2$	Methotrexate Triglutamate
m/z	Mass/Charge
7-OHMTX	7-Hydroxymethotrexate
7-OHDAMPA	4-Amino 7-hydroxy-N^{10}-methylpteroic Acid
R_S	Resolution
$t_{\frac{1}{2}}$	Half-life
V_d	Apparent Volume of Distribution

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DECLARATION

All the work presented in this Thesis was performed by the author, except where otherwise acknowledged.

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SUMMARY

This thesis describes the development of specific assays for two important anti-cancer agents which have been available for some time, methotrexate (MTX) introduced in 1949 and doxorubicin (ADR) introduced in 1969. Both of these drugs undergo significant Phase I metabolism and since such metabolites may be of pharmacological or toxicological significance the assays developed had to be capable of estimating these as well as the parent drugs. High performance liquid chromatography (HPLC) is an ideal technique for this purpose.

The assay developed for MTX and its metabolites employed a liquid/solid extraction which gave reproducibly high recoveries of MTX, its deglutamated metabolites and their 7-hydroxylated derivatives, followed by an HPLC procedure using an anionic ion-pairing agent in the eluant which gave excellent chromatographic efficiency and resolution. This resulted in a highly sensitive and specific assay for all the above components.

The assay was applicable to serum and urine and a modified method applicable to in vitro studies was also developed and used for analyses of cells both bacterial and human, and the culture media in which they were grown.

Studies using clinical samples showed that the assay was sufficiently sensitive to measure MTX and its metabolites in plasma from patients treated with low-dose oral MTX for psoriasis. High-dose studies were carried out during following IV infusions of MTX in cancer patients. The results obtained gave rise to some new findings concerning MTX handling.

In low-dose, intra-individual MTX clearance was highly reproducible, but there was substantial inter-subject variation in this and other pharmacokinetic parameters. In particular MTX bioavailability varied between 17 and 120%. The implications of this and the other variations in disposition are considered.

In the high-dose studies the kinetics were markedly different from the low-dose studies, most probably due to the presence of other drugs and the possibility of third spaces. Sequential high-dose studies showed that changes in the renal component of clearance was a major factor in accumulation of 7-hydroxy methotrexate (7-OHMTX). Clearances were calculated from both urinary data and plasma data and the results were found to be comparable.

A second potential metabolite, DAMPA, which had been reported in plasma by Donehower (1979) was detected in some plasma samples following high-dose MTX therapy. The kinetics indicate that the most likely source of this is as an impurity in the infusion fluid rather than as a result of metabolism in man by hepatic bacterial

deglutamation. This product was not detected in vitro following incubation of either bacterial or human cell lines with MTX.

A third potential metabolite of MTX, 7-OHDAMPA, not previously described in man, was synthesised and characterised in vitro and some evidence obtained as to its presence in urine from patients with DAMPA present in plasma.

An HPLC assay for doxorubicin (ADR) was developed which was highly sensitive for ADR and its main Phase I metabolites. The HPLC assay employed a novel separation mechanism which was fully investigated and an explanation proposed. The assay proved suitable for the examination of clinical samples.

The effects of gastrointestinal bacteria and human cell lines were investigated and metabolism of these drugs in some cells was found, although the relevance of these findings to cell resistance remains to be ascertained.

Similar studies with ADR showed that bacterial cells metabolised the drug but not to any of the known metabolites. These studies indicate that the use of specific methods for measuring metabolites of anti-cancer drugs as well as the parent drug can lead to more information as regards the metabolism and disposition of these drugs than the non-specific tracer methods employed in many reference studies. It is suggested that the

application of these and similar methods to clinical studies in patients treated with chemotherapeutic agents may lead to more rational individual dosage regimes giving rise to increased efficiency and reduced toxicity.

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Cancer is a derangement of normal cell growth. The neoplastic cells are less well differentiated than normal cells and are not subject to the usual control mechanisms; this results in proliferative growth. Such derangement of cell growth can occur in any type of cell from the highly specialised to the general. It is often said that tumour growth is the rapid growth of cells, in some instances e.g. melanoma this is true, but often growth occurs over a long period; it is the uncontrollable nature of growth that characterises these diseases.

Tumours may be broadly characterised as benign and malignant, the former are characterised by slow growth and a lack of spread to other sites; malignancies are more vigorous and are very likely to distribute secondaries from the primary site. Despite the lack of differentiation tumours are capable of producing biologically active proteins that may have e.g. endocrine functions.

In the diagnosis and treating of cancers Clinical Chemists have concentrated on the measurements of 'tumour markers' which have been detected in the plasma or urine of patients with specific tumours and also derangements in metabolism especially those which lead to abnormalities of endocrine function. The majority of these are poor diagnostic tools but some may be used in

prognosis to monitor treatment. Examples are enzymes and various hormones which have been used as tumour markers (; Buckman, 1982; Ruddon, 1982).

Cell Kinetics

All dividing cells follow the cell cycle which has four main phases (Greenwald and Goldstein, 1973). These are:

1. Mitosis

Which consists of prophase in which the chromosome condenses and each chromosome splits into 2 chromatids, each of which contains half of the original DNA and half of newly synthesised DNA, metaphase in which the chromosomes separate and become aligned along micro-tubules which connect the 2 centrioles of the nucleus; anaphase in which the chromatids separate and telophase in which the nucleus and cells divide.

2. GAP_1 (G_1) phase

This phase is variable depending on cell type. Cells which are not rapidly dividing, e.g. normal liver cells, remain in G_1. Cellular growth (in size) occurs mainly in this phase.

3. S-phase

DNA synthesis takes place in S-phase. It has a relatively constant duration in mammalian cells (6-8 hours).

4. GAP_2 (G_2) phase

In this phase, like G_1, the undivided cells are quiescent for a fairly constant time. In mammalian cells the average is about 2 hours.

This is the cell cycle and the time taken for one cycle is called the generation time. The generation time for human marrow cells (the most rapidly dividing) is slightly less than 24 hours.

The Behaviour of Cancer Cells

Cancer cells differ from normal cells in three ways (Wood and Strauli, 1973). These are:

1. Uncontrolled growth.
2. Invasion

   Metastatic formation of some of the following:

   (a) Enzymatic or other lytic factors that break down normal tissues.

   (b) Tissue pressure caused by tumour growth.
3. Spread

   Tumour cells spread and form metastases by moving via both the blood vessels and the lymphatic system.

Medical treatment of cancer in all its forms is usually by radiotherapy, chemotherapy or a combination of these. Few of these procedures are highly effective and there are many side-effects.

During the last 40 years a wide range of anti-cancer drugs have been developed each of which have known effects on the growth of malignant cells.

Drug Classification (Calman, Smyth and Tattersall, 1980)

A rough classification of the anti-neoplastic drugs divides them into the following groups.

1. Cell cycle (non-specific)

These drugs kill malignant and normal cells at any stage in the cell cycle or when quiescent. An example is nitrogen mustard (γ radiation works in the same way).

2. Cell cycle (phase-specific)

The major drugs in this class are thymidine, vinblastine, vincristine, methotrexate and cytosine arabinoside. These drugs are toxic to cells in particular phases of the cell cycle.

3. Cell cycle (non phase-specific)

The drugs in this class are cyclophosphamide, actinomycin D and the nitrosoureas. These drugs are toxic to the cells during periods of growth in the cell cycle, but not when quiescent i.e. G_1 and G_2.

The Effect of Anti-Cancer Drugs

All of these drugs may be effective against one type of tumour but ineffective against others. Continuous and long term research is underway in order to select the most appropriate regimens. At present there are a few tumours for which chemotherapy has been shown to provide a cure, these include acute lymphocytic leukaemia in

children, Burkitt's lymphoma, choriocarcinoma in women, and Hodgkin's disease. Some tumours are highly sensitive to chemotherapy giving long remissions, examples are chronic lymphocytic leukaemia, lymphomas, ovarian carcinoma, testicular teratoma, some head and neck tumours and acute myeloid leukaemia.

A further group of tumours are sensitive to chemotherapy but have limited remission. These include osteogenic sarcoma, myeloma, gastric carcinoma, bladder tumours and prostatic carcinoma. Chemotherapy has not so far been shown to be effective against non-small cell bronchogenic carcinoma, carcinoma of the pancreas, colorectal carcinoma or melanoma.

The ideal drug for the treatment of any individual cancer is one which is capable of penetrating a tumour and eliminating all malignant cells. It should also be free of acute or chronic side-effects. It is a fact that none of the drugs fit these criteria.

The toxicity of the anti-cancer drugs is due to the fact that they exert their effect on normal as well as malignant cells. Drugs which are active against cells which are rapidly dividing in fast growing tumours are also active against normal tissues with a high rate of turnover e.g. gut and blood cells. As an example of the latter the toxicity of some anti-neoplastic drugs to the bone marrow is illustrated in Table 1 (Calman, Smyth and Tattersall, 1980).

TABLE 1

Marrow Toxicity of some Anti-Cancer Drugs

Drug	Anaemia	Neutropenia	Thrombocytopenia
Methotrexate	+	++	++
Mercaptopurine	+	++	++
Fluorouracil	+	+	+
Cytosine arabinoside	+	++	++
Cyclophosphamide	+	++	+
Melphalan	+	++	++
Chlorambucil	+	+	+
Cis-platinum	++	+	+
BCNU/CCNU	+	+	++
Adriamycin	+	++	++
Actinomycin	+	++	++
Bleomycin	±	±	±
Vincristine	±	±	±
Vinblastine	+	++	+

Combined regimes of two or more anti-neoplastic drugs are used in the treatment of most tumours. Some of these combinations have been found to be highly effective, however, interaction occurs both between chemotherapeutic agents and with other drugs which may be co-prescribed. Some of the better known interactions have been described by Stuart and Stockley (1979).

Therapeutic drug monitoring is becoming increasingly used for studying the disposition of drugs in patients under treatment in order to avoid toxicity, and to study the disposition in patients in order to maximise effect (Richens and Marks, 1982).

Chemotherapeutic drugs seem to fit the criteria which determine whether a drug should be measured. These are:

1. The drugs require to be given at doses near or within the toxic range in order to be effective i.e. a low therapeutic index.
2. They are administered to patients who may have abnormal "metabolism distribution" (fluid compartments) or excretion.

The majority of studies of the effectiveness and/or toxicity of chemotherapeutic drugs have been concerned only with dosage. Measurements of plasma concentrations of drugs are increasing in clinical trials, but it is true to say that only for methotrexate (MTX) has an

established protocol been developed which relates plasma concentrations to the need for treatment with leucovorin. One example of this protocol states that leucovorin rescue should be initiated when, following high-dose therapy, the MTX plasma concentration exceeds 10^{-5}M at 24 hours, 10^{-6}M at 48 hours or 10^{-7}M at 72 hours. The level of potentially toxic metabolites are not taken into account (Nirenberg et al, 1977).

Part of the reason why more drug monitoring is not performed during chemotherapy is the lack of rapid specific methods for determining the drugs themselves and their active metabolites. In addition many studies of chemotherapeutic drugs use either radiotracer-labelled drugs in order to study the disposition, in which case it is not possible to differentiate between drug and metabolite or use other non-specific methods e.g. total fluorescence.

There are few reported studies in which specific measurements of drug and metabolite levels have been made during treatment.

In the case of doxorubicin (ADR), there is as yet no directly agreed link between plasma drug concentrations and toxicity. Again studies on metabolite concentrations in patients are few.

The aim of this thesis was therefore the development of HPLC methods, which have the advantage of specificity, for the measurement of MTX and its metabolites and ADR and its metabolites, and to study the source of these metabolites and their kinetics in vivo.

CHAPTER 2

METHOTREXATE

INTRODUCTION

HISTORY

The folate analogue aminopterin, an inhibitor of the enzyme dihydrofolic acid reductase (DHFR), essential for the synthesis of DNA was first discovered and investigated by Farber et al (in 1948), who demonstrated the effectiveness of aminopterin in children suffering from acute leukaemia; 10 out of 16 children responded with a temporary remission. However aminopterin is highly toxic, and this led to a search for a less toxic analogue. Methotrexate (amethopterin) was proposed by Farber in 1949.

Leucovorin (folinic acid) was prepared in 1950 and its biological and chemical properties were studied (Broquist, Stokstad and Jukes, 1950). Leucovorin was found also to act as a substrate for DHFR, by which it is converted to tetrahydrofolic acid (Figure 1), thus preventing and reversing the toxic effect produced by methotrexate (MTX) and aminopterin (Schoenbach, Greenspan and Closky, 1950).

STRUCTURE

The structures of MTX, folic acid, leucovorin and aminopterin are shown in Figure 2. Aminopterin (4-amino pteroylglutamic acid) differs from folic acid by an amino group replacing the hydroxyl group at the 4-position.

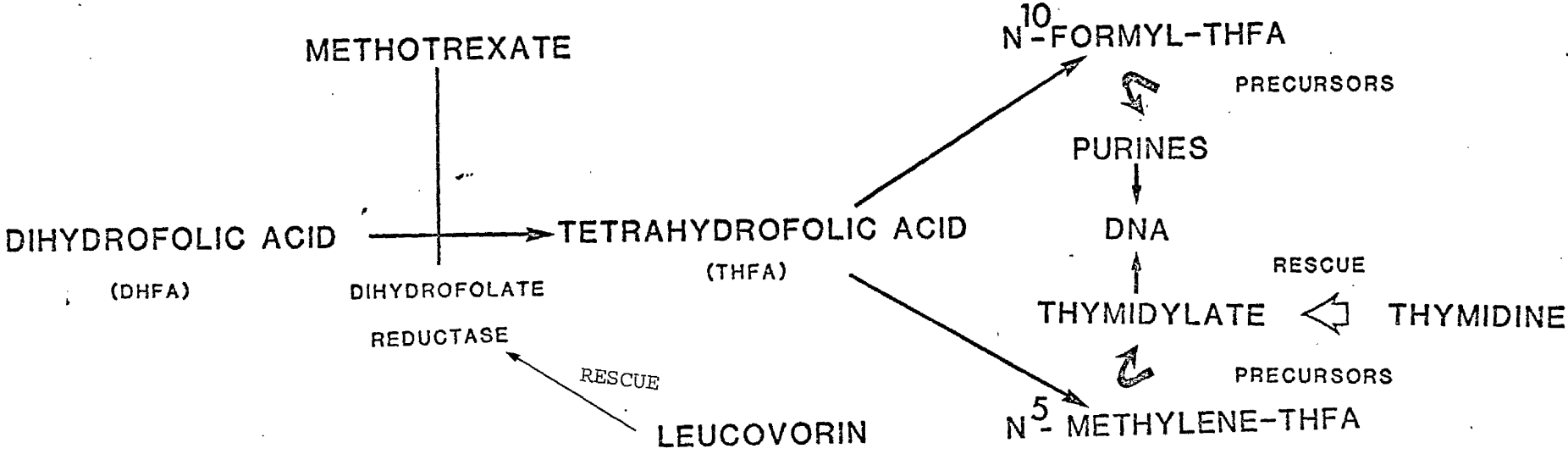
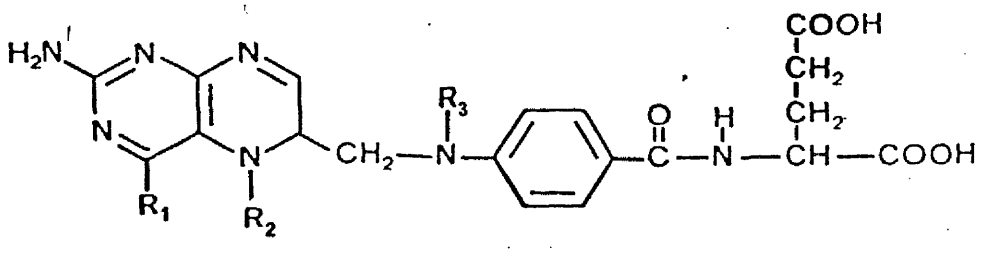


FIGURE 1 Mechanism of action of Methotrexate and Leucovorin-rescue.



	R_1	R_2	R_3
Methotrexate	NH_2	—	CH_3
Folic acid	OH	—	H
Leucovorin	OH	CHO	H
Aminopterin	NH_2	—	H

FIGURE 2 The structure of Methotrexate and its analogues.

Methotrexate (4-amino 4-deoxy N^{10} methylpteroylglutamic acid) differs from aminopterin by methylation at the N^{10} position.

Leucovorin (folinic acid) is N^5 formyltetrahydrofolic acid and differs from folic acid by an aldehyde group at N^5 position.

MECHANISM OF ACTION

DHFR is the enzyme responsible for converting dihydrofolic acid to tetrahydrofolic acid (Figure 1). MTX, like aminopterin, inhibits the reaction by reversibly competing with dihydrofolic acid thus disrupting the folate cycle which produces the C_1 fragments necessary for the production of both purine nucleotides and thymidylate and consequently DNA synthesis.

Leucovorin reverses the inhibitory effect of MTX since it is converted in vivo to 5-methyltetrahydrofolic acid and then to tetrahydrofolic acid (Figure 1) (Bleyer, 1978) thereby replacing the pool of C_1 group donors. Since MTX and leucovorin compete for the enzyme (Burchenal and Babcock, 1951) the relative concentrations of MTX and leucovorin are important.

Thymidine has also been used as a rescue agent in patients treated with MTX since it is converted to thymidylate, the major pyrimidine precursor for DNA synthesis (see Figure 1). The supply of purine nucleotides is not completely removed during DHFR inhibition since these are also synthesised in bone

marrow cells which utilise predominantly preformed purines derived from the liver (Schornagel et al, 1978), but there is no comparable scavenging system by which thymidylate can be converted directly to the nucleotide.

CLINICAL ASPECTS OF METHOTREXATE

Methotrexate (MTX) is used in the treatment of a variety of malignancies. Low doses are effective in choriocarcinoma (Hertz, Lewis and Lipsett, 1961), acute lymphocytic leukaemia (Freeman, Wang and Sinks, 1977) and breast carcinoma (Yap et al, 1979).

In high dosage, the drug is effective against malignant lymphoma (Djerassi and Kim, 1976), osteogenic sarcoma (Jaffe et al, 1974; Jaffe et al, 1977), carcinoma of the head and neck (Khandekar and Wolff, 1977; Vogler at al, 1979) and small cell carcinoma of the lung (Djerassi et al, 1972; Pitman and Frei, 1977).

MTX is usually used in conjunction with other anti-neoplastic drugs either concomitantly or sequentially. A review was published recently (Cancer Treatment Reports 1981

Dosage

MTX was first used clinically for acute lymphocytic leukaemia at an oral dose of 1-5 mg/m^2 per day.

In the early 1960's intermittent parenteral MTX at 30 mg/m^2 twice weekly was observed to be more effective for the treatment of childhood acute lymphocytic leukaemia (Selawry and James, 1965). Solid human tumours resistant to these doses were shown to respond in many

instances to short infusions (about 4 hours) of higher doses in the range of 100-200 mg/m^2 (Djerassi, 1967). Recently massive doses of MTX in the range of 3-30 gm/m^2 in conjunction with leucovorin-rescue have been administered (Djerassi et al, 1972). These large doses were administered as an intravenous bolus or as an infusion from 4-42 hours in duration (Hande, Donehower and Chabner, 1978). In addition to variation in the dose infused, the dose of leucovorin was also varied from 3-40 mg/m^2 given by the IV, IM or oral routes every 6 hours commencing at the end of infusion until the observed plasma level of MTX achieved was less than the toxic level (Sadee, 1980). The level associated with risk of MTX toxicity after 24, 48, and 72 hours is shown in Table 2.

Toxicity

During MTX therapy, there are a number of toxic side-effects, some of which are related to folate stress and may be life threatening (Vogler et al, 1979). The main manifestations include:-

1. Hepatotoxicity

Hepatic fibrosis was found in 11 of 43 patients treated with MTX for psoriasis (Robinson et al, 1980) and 11 out of 44 patients by Dahl, Gregory and Scheuer (1972), cirrhosis was found in another 6 patients (Robinson et al, 1980). The prevalence of cirrhosis and fibrosis was significantly greater in patients treated

TABLE 2

Shows the toxic level and the toxicity risk after 24, 48, 72 hours (Nirenberg et al, 1977)

Time After Administration	Hours		
	24	48	72
MTX Plasma levels (molar)	10^{-5}	10^{-6}	10^{-7}
Toxicity risk: number patients/all patients with high MTX level	28/74	25/68	20/90

using frequent small doses (2.5 mg orally daily for between 2-5 days) than in those treated with intermittent large doses (10-25 mg either orally or intramuscularly at intervals of 1-4 weeks). Hepatic fibrosis and cirrhosis have been attributed to treatment with MTX in patients with leukaemia (Colsky, Greenspan and Warren, 1955; Hutter et al, 1960). Less severe abnormalities of liver histology were reported in patients treated for benign ocular disease (Hersh et al, 1966). Liver cirrhosis or fibrosis can only be ascertained by liver biopsy since liver function tests are not diagnostic (Weinstein, 1973).

2. Pulmonary complications

Pulmonary complications have been reported in patients treated with MTX for acute leukaemia (Clarysse et al, 1969; Robertson, 1970; Rawbone et al, 1971; Everts, Westcott and Bragg, 1973) and skin diseases (Filip et al, 1971; Goldman and Moschella, 1971).

Although MTX pneumonitis has not been considered dose-related (Clarysse et al, 1969; Goldman and Moschella, 1971; Rosenow, 1972; Everts et al, 1973) it has never been reported in patients receiving less than 20 mg per week. The syndrome occurred in patients who had been treated with the drug for periods ranging from 12 days-5 years (average 138 days). The total dose received was between 40 and 6500 mg. Some patients were

also receiving corticosteroids at the time that the pulmonary disease developed (Goldman and Moschella, 1971).

3. Neurotoxicity

Severe neurotoxicity has been reported following MTX administered intrathecally (IT) (Wilson and Norrell, 1969; Norrell et al, 1974). Of 231 patients, 13 were found to have leucoencephalopathy after IV and IT MTX administration (Price and Jamieson, 1975); 9 out of 20 patients developed leucoencephalopathy after IV high-dose MTX (Aur et al, 1978).

Recently Bowles et al (1981) reported that no abnormalities of the brain were found in 18 patients suffering from osteosarcoma treated with high-dose MTX and leucovorin-rescue. The level of MTX in cerebrospinal fluid after intrathecal administration averaged 13.8 times that of the serum concentration (1.7×10^{-7}M) (Bleyer, Drake and Chabner, 1973). These observations suggest that the neurotoxicity associated with intrathecal MTX is due to prolonged exposure to a high concentration of drug in the tissues of the central nervous system.

4. Renal toxicity

Link et al (1976) suggested that high-dose MTX therapy regularly produced a reversible decline in glomerular filtration rate (GFR); if sufficiently severe the local renal toxicity may contribute to development of systemic toxicity due to inadequate clearance of MTX.

The same authors reported a correlation between lowered creatinine and inulin clearance and MTX-induced renal toxicity. A case of fatal renal failure in a patient treated with MTX showed severe histological damage (Ahmed, Shen and Bleyer, 1978).

Changes in plasma urea correlated with clinical indices of renal function, the urea rising as the other symptoms progressed. In those patients who recovered, the urea returned to normal. In patients who developed severe toxicity or in some patients with only moderate toxicity, decrease in the renal clearance of both inulin and PAH occurred (Condit, Chanes and Joel, 1969).

The complication of acute renal failure with high doses of MTX is particularly serious because MTX is mainly eliminated (> 90%) by the kidneys (Jaffe and Paed, 1972). For renal damage due to crystallisation of metabolites see Renal Excretion, page 22.

5. Bone marrow toxicity

In sequential bone marrow biopsies of 3 patients receiving high-dose MTX with leucovorin-rescue, initial accumulation of cells in G1-S phase (Day 1) were followed

by a significant proliferative response (Days 4-7) and return to pretherapy values (Krishan et al, 1976) in one patient, in bone marrow samples of the third day, 90% of the cells were blasts. At Day 7, 10% of the cell in the bone marrow were blasts.

Avoidance of Methotrexate Toxicity

The toxicity of MTX with and without leucovorin was studied in one patient, who, when given 2.5 mg MTX daily for 8 days, developed mouth ulceration and leukopenia, however doses of 45-60 mg daily for 21 days were tolerated when covered by simultaneous parenteral administration of 3 mg leucovorin (Burchenal and Kingsley-Pillers, 1951).

Toxicity of MTX to organs such as bone marrow is dependent on both the duration of exposure to the drug and its extracellular (plasma) concentration. Concentrations of leucovorin of 10^{-7}M, 10^{-5}M and 10^{-3}M are required to rescue normal cells exposed to a level of MTX of 10^{-7}M, 10^{-6}M and 10^{-5}M respectively (Pinedo et al, 1976). The administration of MTX must be stopped immediately if toxicity occurs. The use of thymidine to restore bone marrow activity has been claimed to give better results than leucovorin (Howell, Krishan and Frei, 1979). MTX-induced pulmonary changes have been treated by administration of prednisone (Schwartz and Kajani, 1969) and by daunorubicin (Pasquinucci, Ferrara and Castellari, 1971).

Renal damage is avoided by prehydration of the patient and the administration of bicarbonate to produce an alkaline urine as MTX and its metabolites are acidic compounds and alkali increases their solubility in urine (Hande et al, 1978).

PHARMACOKINETICS

Absorption

1. Oral

Doses of MTX below 30 mg/m^2 are well absorbed with peak serum levels appearing after 1-2 hours. Slow absorption of small doses (peak after 4 hours) was seen in 5 out of 18 patients studied (Freeman-Narrod, 1962). The levels were determined by a fluorimetric method. In half of the patients they also found a definite slowing in the rate of absorption over a 6 week course of therapy. With doses in excess of 80 mg/m^2, the extent of absorption was reduced by 50-70% (Henderson, Adamson and Oliverio, 1965; Wan et al, 1974). The kinetics were studied following the administration of radiolabelled MTX.

McVie et al (1981) studied high-dose oral MTX. 250 mg of MTX in divided oral doses (50 mg each dose) every 2 weeks was administered to 9 patients followed by 15 mg leucovorin four times daily for 2 days. The biological specimens were analysed by enzyme immunoassay

(EMIT). Although the area under curve was always less than the equivalent IV dose, there was no evidence of malabsorption of the drug.

2. Intramuscular administration (IM)

The gradual absorption of drug from the IM site offsets the rapid fall in serum levels during the initial distribution phase (Freeman-Narrod et al; 1975) a fluorimetric assay was used.

3. Intrathecally (IT)

After IT injection of MTX absorption from the central nervous system, equilibration into the plasma compartment proceeded slowly. IT administration of 10-15 mg/m^2 resulted in plasma levels above $10^{-8}M$ for 2-3 times longer than after the same doses administered intravenously (Jacobs et al, 1975). The authors used a DHFR inhibition method.

4. Intravenous and infusion administration

This is preferred as very high doses can be administered by this route.

Distribution

After IV administration the disappearance of MTX from plasma is triphasic (Bleyer, 1978), the first half-life ($t_{\frac{1}{2}}\alpha$) is due to distribution and the second half-life ($t_{\frac{1}{2}}\beta$) is associated to renal clearance. The third half-life ($t_{\frac{1}{2}}\gamma$) is thought to reflect the entero-hepatic circulation of MTX (Bleyer, 1978). After an IV

dose of 80 mg/m^2 MTX is concentrated in liver with liver:plasma ratios of 4:1 at 3 hours and 8:1 at 24 hours.

MTX is readily transported into human skin after parenteral administration (Comaish and Juhlin, 1969) but not after topical application (Fry and McMinn, 1967; McCullough and Weinstein, 1974).

Distribution of MTX into cerebrospinal fluid, plasma and peritoneal cavities occurs slowly and if these spaces are pathologically increased as in ascites or pleural effusion, they act as a reservoir prolonging the presence of MTX in the plasma compartment (Wan et al, 1974).

Intra-arterial injection of the MTX leads to higher tissue levels in the region supplied by the artery than after intravenous injection (Anderson et al, 1970). These slightly higher levels are probably clinically insignificant in comparison to the substantial risks of intra-arterial infusion.

1. Plasma protein binding

In man, 45-51% of MTX in plasma was found to be protein-bound (Henderson et al, 1965; Wan et al, 1974) using ultrafiltration to separate bound and free drug. Leigler et al (1969) and Taylor and Halprine (1977) calculated that 70% of MTX was bound in plasma. Steele et al (1979) estimated the plasma protein binding for MTX in vitro by a radioimmunoassay measurement on ultrafiltrate, in which continuous filtration overcame many of

the membrane binding problems. The percentage of MTX bound to serum protein remained constant at 95.11%±2.26 (SD) over the range 1 x 10^{-6}M to 3 x 10^{-5}M. No great change in the protein binding occurred until the serum concentration exceeded 5 x 10^{-5}M. At a concentration greater than this the free drug proportion increased much more rapidly than the total serum concentration.

These differences reflect the non-comparability of different methods of studying protein binding. Highly bound acidic compounds such as aspirin and para-amino-hippurate can displace MTX from its plasma protein binding sites (Leigler et al, 1969).

Metabolism

The known routes of metabolism of MTX in man are shown in Figure 3. The major metabolite of MTX is 7-hydroxymethotrexate (7-OHMTX). 7-OHMTX was isolated and identified in human urine after high-dose therapy (> 50 mg/kg) (Jacobs et al, 1976).

Jacobs et al (1977) reported that 7-OHMTX was not observed in patients following low-dose therapy (< 10mg/kg) and suggested that the production of this metabolite was dose-dependent. However Chan et al (1980) reported that 7-OHMTX production was not dose-dependent and was found in human plasma in patients receiving such doses.

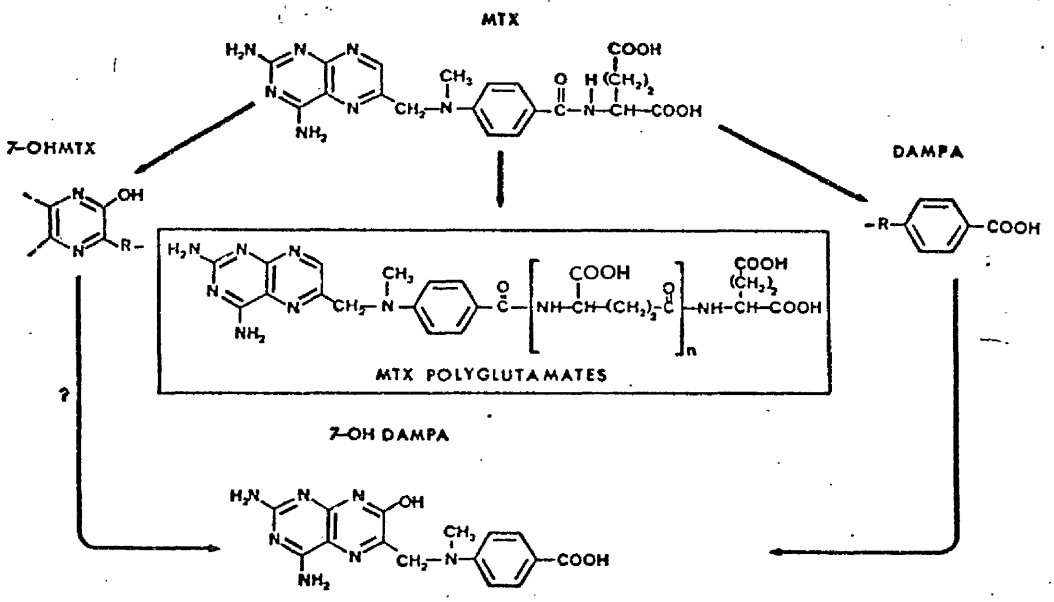


FIGURE 3 The metabolic pathway of Methotrexate.

7-OHMTX has itself been shown to be an inhibitor of mammalian dihydrofolic acid reductase in vitro (Jacobs et al, 1977); although it is 200-fold less potent than MTX. It is also 4 to 5-fold less water soluble than the parent compound, and may cause renal damage due to crystallisation in the tubules (Jacobs et al, 1976).

4-Amino-4-deoxy N^{10} methylpteroic acid (DAMPA or APA) is produced by the removal of the glutamate residue from MTX by cleavage of the pteroate-glutamate peptide bond; it has been suggested that it is produced by the carboxypeptidase of some intestinal bacteria (Donehower et al, 1979), during enterohepatic circulation of MTX. However it may also be present as a contaminant in some IV preparations (Chatterji, Frazier and Gallelli, 1978).

DAMPA is also much less soluble than MTX; the limit of solubility of this compound suggests the possibility that renal precipitation may occur (Donehower, 1980).

DAMPA is a potent inhibitor of dihydrofolic acid reductase although the concentrations present in plasma are likely to be low (Kessel, 1969).

Theoretically, DAMPA could be metabolised in a manner similar to the oxidation of MTX giving rise to 7-hydroxy-4-amino-4-deoxy-N^{10}-methylpteroic acid (7-OH-DAMPA) (Valerino et al, 1972) as a further metabolite.

The major intracellular metabolites of MTX are the polyglutamates (Hendel, 1978). Following passage into cells, MTX is converted to a series of polyglutamates by

the addition of glutamyl residues. These may be retained within the tissues for up to 3 months before being degraded by pteroylglutamyl-γ-glutamyl carboxypeptidase and excreted via both the intestinal and the renal routes as MTX (Baugh, Krumdieck and Nair, 1973). Nair and Baugh (1973) synthesised a series of 6 MTX polyglutamates ranging from MTX diglutamate (MTXG1) to MTX hepata-glutamate (MTXG6), andelevated their biological activity.

MTXG1 and MTXG2 have been found in human red blood cells (Baugh et al, 1973) while MTXG1 has been found in human liver cells (Jacobs et al, 1977); MTX polglutamates are also strong inhibitors of dihydrofolic acid reductase activity (Hendel, 1978).

Excretion

1. Renal excretion

The major route of elimination of MTX at low-dose (< 30 mg/m^2) is renal excretion (> 80%) (Huffman et al, 1973). About 50% of infused high-dose MTX was recovered in a 6-12 hour collection of urine post infusion (Stoller et al, 1975). Renal clearance of MTX in 15 patients with steady-state plasma levels ranging from 2 x 10^{-7}M to 4 x 10^{-6}M was between 144-217 ml/minute with a mean of 179. This indicates that MTX is not only filtered but also actively secreted by the renal tubules and that MTX clearance is not directly related to the level of free MTX presented to the kidney (Liegler et al, 1969).

It has been suggested that renal secretion might be reduced by co-administration of salicylate or para-aminohippurate (Leigler et al, 1969) giving a higher plasma concentration achieved for the same dosage, however this is balanced increased free MTX in the GFR. Pretreatment of monkeys with probenecid blocks renal tubular secretion of MTX (Bourke et al, 1975) and this property of probenecid has been exploited in order to slow elimination of MTX, thereby allowing a reduction in dosage.

The low solubility of MTX in urine creates a risk of crystallisation in the intratubular lumen. Solubility of MTX falls from 20 mM to 2 mM when the pH of the medium is reduced from 6.9-5.7 (Stoller et al, 1975). As shown in Table 3 the solubility of 7-OHMTX and DAMPA are much lower than MTX so there is also a possibility of renal damage due to precipitation of these metabolites in the renal tubules (Donehower, 1980) despite their reported lower plasma concentrations.

2. Biliary excretion

In man 6.7-9% of the administered dose has been recovered by duodenal aspiration during the first 24 hours. Only a trace amount of MTX (i.e. less than 2% of the dose) was recovered in the 24-48 hour period of bile collection (Shen and Azarnoff, 1978).

TABLE 3

Aqueous solubility of MTX and Metabolites

Compound	mM		
	pH5	pH6	pH7
MTX	0.85	3.4	19.89
7-OHMTX	0.27	0.78	3.3
DAMPA	0.15	0.31	2.6

3. Extra-renal excretion

The ratio of breast milk MTX to plasma MTX concentration was 0.08:1 (Johns et al, 1972). A Low level of MTX in saliva was found following 30 mg/m^2 IV dose of MTX (Huffman et al, 1973).

METHOTREXATE ASSAYS

Due to the wide use of MTX and high risk of toxicity, it is essential to monitor the level of the drug in the plasma during and following therapy (Bertino and Isacoff, 1978). Several techniques have been used for measurement of MTX in biological fluids.

Biological Assay

Such techniques depend on inhibition of growth of streptococcus faecalis by incubation with MTX over 24 hours. The concentrations of MTX are calculated from the relative diameter of the zone plotted against the logarithm of the concentration of drug (Burchenal et al, 1951). Although this method has a sensitivity of 2×10^{-8}M, it has a poor specificity because other substances in biological fluids with growth inhibitory properties may give falsely elevated results. The precision is $\pm$12% and the assay is time consuming. Results are seldom available fast enough to influence the rescue procedure which is decided on clinical criteria.

Fluorimetric Methods

A fluorimetric method for the determination of MTX in biological fluids was first reported by Freeman (1957). Following protein precipitation by trichloroacetic acid, potassium permanganate solution was used to oxidise MTX to 2,4-diamino pteridine 6-carboxylic acid (Figure 4). The sensitivity of the method was 1 x 10^{-7}M but it had poor specificity as metabolites yielded an identical fluorescent product.

Chakrabarti and Bernstein (1969) modified the Freeman method. A protein precipitation step including heating significantly improved the recovery by releasing bound MTX from the precipitant. Despite the increased recovery the sensitivity of this method was the same as Freeman's (1 x 10^{-7}M) and suffered from the same problem of low specificity.

Recently an improved spectrofluorimetric method was reported using sodium bisulphite instead of hydrogen peroxide and Tris buffer pH 8.5 instead of phosphate or acetate buffer pH5 in the oxidation procedure (Kinkade, Vogler and Dayton, 1974). In addition a long extraction procedure was employed. The method was linear over a wide range of plasma concentrations but had a sensitivity of only 2 x 10^{-7}M of MTX in plasma despite a 10-fold improvement in fluorimetric intensity over the Freeman assay. The method had high recoveries (93%) and was highly reproducible.

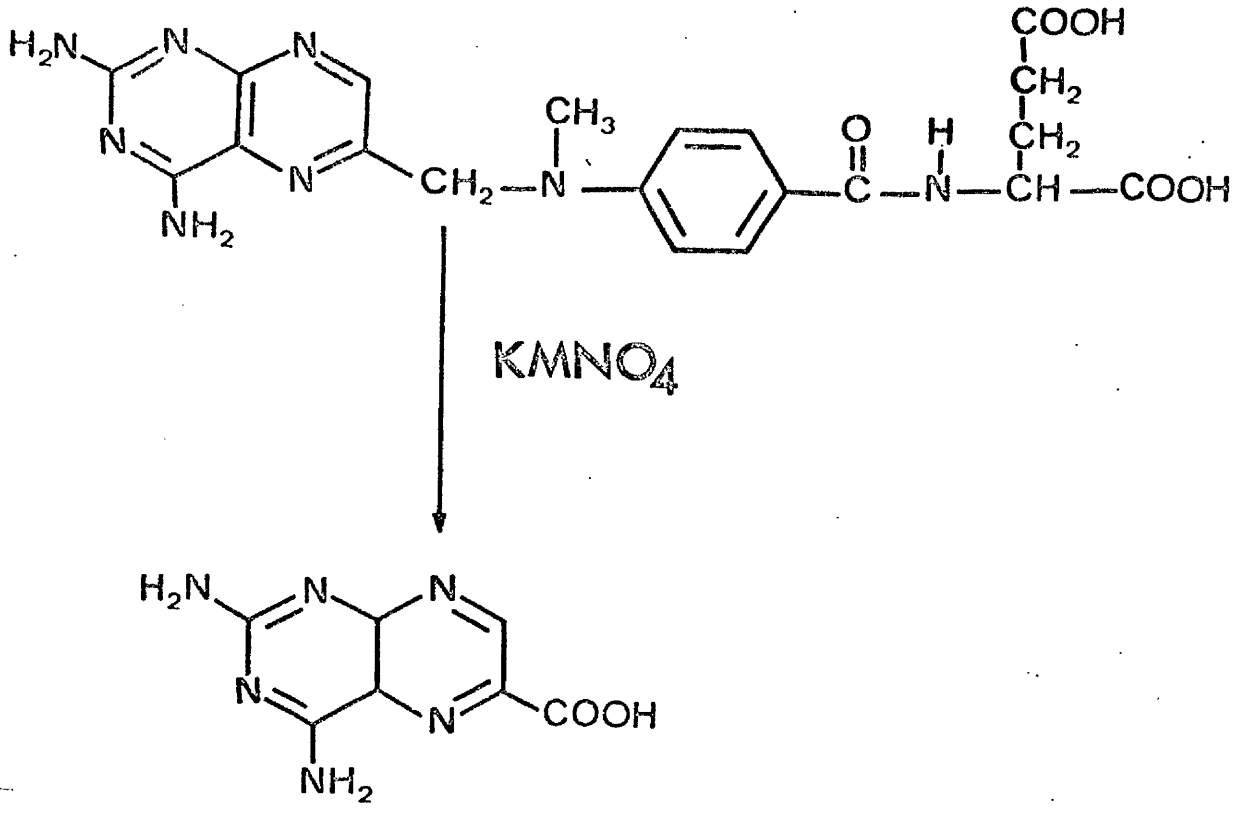


FIGURE 4 The oxidation of MTX to yield a fluorescent product.

Generally the fluorimetric methods lack specificity due to measurement of MTX metabolites.

Dihydrofolic Acid Reductase Inhibition Methods

The decrease in enzyme activity following inhibition by MTX may be measured by following the decrease in absorbance at 340 nm as NADPH is oxidised to NADP during reduction of dihydrofolic acid to tetrahydrofolic acid (Bertino and Fischer, 1964). The sensitivity of this method is 2×10^{-8}M but there is interference with MTX metabolites or reduced folates.

Rothenberg (1965) produced a radioenzymatic assay for MTX using folic acid reductase (obtained from chicken livers) to reduce the tritiated folic acid to tetrahydrofolic acid. Addition of MTX to the incubation mixture inhibited the reaction. From this MTX could be quantitated and standardised.

Falk et al (1976) produced an enzymatic assay for MTX in serum and cerebrospinal fluid based on inhibition by MTX of DHFR from bacteria. The quoted sensitivity was also 2×10^{-8}M and again specificity was not reported.

Radioimmunoassay

Radioimmunoassays have been developed for MTX (Levine and Powers, 1974) using either rabbit or goat antisera raised against MTX-hemocyanin conjugate. As would be expected these assays are highly sensitive, having a limit of detection of 2×10^{-9}M; the methods lack specificity and have not achieved widespread usage.

Hendel, Sarek and Hvidberg (1976) used MTX conjugated to methylated bovine serum albumin. The sensitivity of their optimised assay was 4 x 10^{-9}M, there was little cross-reactivity with folic acid, leucovorin or tetrahydrofolic acid. The authors did not test for cross-reactivity with MTX metabolites.

Aherne, Piall and Marks (1977) developed a radio-immunoassay method for MTX using an MTX-ovalbumin conjugate as antigen. This method has been in use for 4 years, during which time the label has been changed from tritium to ^{75}Se (Aherne, Piall and Marks, 1978) and finally to a ^{125}I-MTX (Kamel and Gardner, 1978).

The sensitivity of the method was 2 x 10^{-9}M. The quoted specificity gave 31% cross-reactivity with DAMPA but less than 1% for 7-OHMTX.

Enzyme Immunoassay

A homogeneous enzyme immunoassay for MTX in plasma was developed by Gushaw and Miller (1978) and is now marketed as an EMIT kit by the Syva Corporation. The method is illustrated in Figure 5. The diluted plasma sample is mixed with a reagent which contains antibodies to MTX together with substrate for the enzyme glucose 6-phosphate dehydrogenase (G6PDH). Binding occurs to any MTX or metabolite in the serum or plasma which is recognised by the antibody. A second reagent containing MTX labelled with the enzyme G6PDH is then added. The labelled drug combines with any remaining unfilled

Fig 5

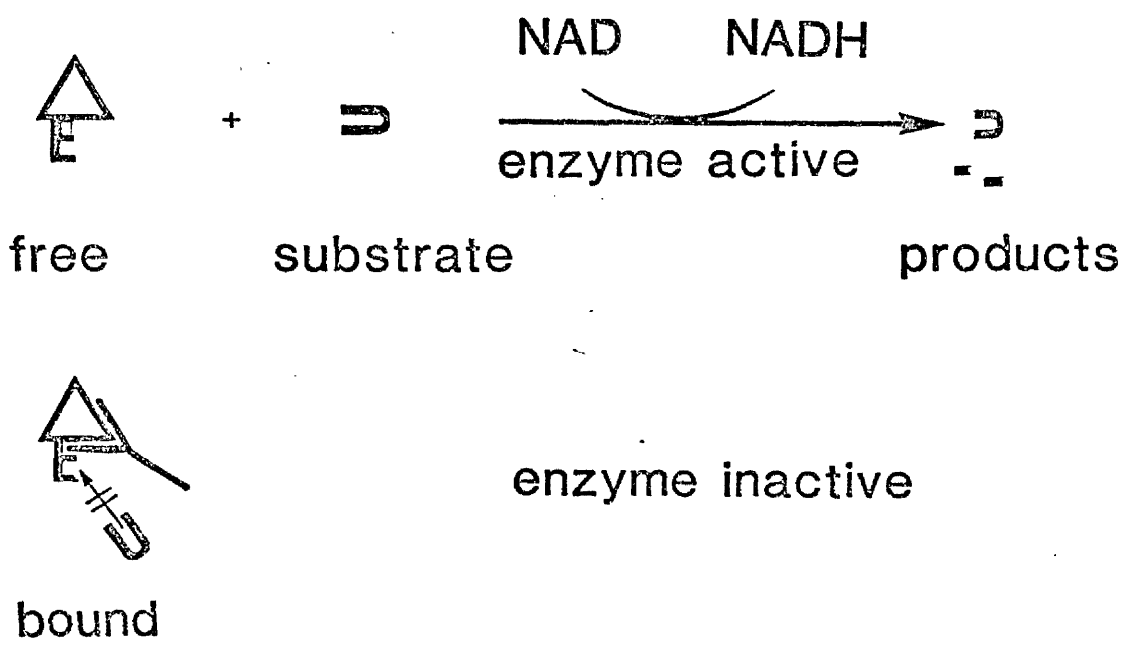


FIGURE 5 The mode of action of a homogenous enzyme immunoassay for a drug.

E = Enzyme

Δ = Drug

⟩— = Antiserum

antibody binding sites, and the enzyme activity is thereby proportionately reduced, as the antibody-bound enzyme drug conjugate is inaccessible to the substrate. The residual enzymatic activity is directly related to the concentration of MTX present in the serum or plasma. The active enzyme converts nicotinamide adenine NAD to NADH, resulting in an absorbance change that is measured spectrophotometrically at 340 nm. Interference from serum G6PDH activity is avoided by use of NAD which functions as a coenzyme only with the bacterial (Leuconostoc Mesenteroides) enzyme used in the assay and not with human G6PDH which requires NADPH.

The sensitivity of the method is 2 $x10^{-7}M$, however specificity is not absolute due to cross-reactivity of the antiserum with some metabolites, especially DAMPA (Gushaw and Miller, 1978).

Double Antibody Enzyme Immunoassay for Methotrexate

Marks et al (1978) produced an enzyme - immunoassay for measuring MTX in plasma by conjugating beta-D-galactosidase to MTX by means of a mixed anhydride reaction and used a double-antibody immunoassay. The quoted sensitivity was 4 x $10^{-9}M$ with a coefficient of variation of 4-15%. There was 40% cross-reactivity with DAMPA. The disadvantage of this assay was the need for overnight incubation. Al-Bassam et al (1979) improved this assay by using a precipitated complex of first and second antibodies which markedly increased the speed of

the assay. The improved assay had a sensitivity of 2×10^{-9}M but the cross-reactivity with DAMPA remained a problem.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been used for the analysis of MTX and other anti-neoplastic drugs. Montgomery et al (1977) reported a reverse-phase HPLC procedure for MTX and related folate analogs, however MTX metabolites were not examined.

Nelson et al (1977) used HPLC with fluorescence detection to estimate MTX in biological fluids following oxidation of MTX by potassium permanganate solution (see Figure 4). 2-Hydroxyfolic acid (fluorescent oxidation product 2,4-dihydroxypteridine 6-carboxylic acid) was used as internal standard. The sensitivity of this method was 2×10^{-8}M. MTX in the trichloroacetic acid extract could be measured with a UV detector at 305 nm, but the sensitivity of UV detection was 8×10^{-7}M. The calculated recovery was only 40%. The authors did not study interference by MTX metabolites but these would be expected to interfere as a non-selective oxidation procedure was used.

Lankelma, Kleijn and Termond (1978) reported an assay for MTX and 7-OHMTX by HPLC in which a short reverse-phase column was used to concentrate the sample, this was eluted with tap water on to an anion-exchange column.

Cerebrospinal fluid (CSF) and plasma were deproteinised by adding a solution of trichloroacetic acid in 0.1 M hydrochloric acid. The sensitivity was 2 x 10^{-8}M but recovery was suboptimal (< 70%). The precision was 5% at a concentration of 4 x 10^{-8}M (n = 3).

The authors did not report on the interference by MTX metabolites and no internal standard was used.

Watson, Cohen and Chan (1978) separated and determined MTX and its metabolite 7-OHMTX in human plasma using anion exchange chromatography. Detection was at 254 nm using N-[4[[(2,4-diamino-6-quinazolinyl) methylamino]benzoyl]] aspartic acid as an internal standard. Perchloric acid deproteinisation with ammonium sulphate, followed by extraction with an organic solvent was used for sample clean-up.

The quoted sensitivity was 2 x 10^{-7}M or 1 x 10^{-7}M using detection at 254 nm or 315 nm respectively. The precision at 1 x 10^{-5}M was 3% (n = 8). Leucovorin and folic acid did not interfere with this assay. Twenty-six per cent recovery for 7-OHMTX was seen under these assay conditions. The authors did not discuss other interferences.

Donehower et al (1979) reported an HPLC assay that allowed determination of DAMPA, as well as MTX and 7-OHMTX following an anion-exchange extraction procedure.

Two HPLC column systems were used, a preparative column (30 cm x 0.78 cm ID) and an analytical column (30 cm x 0.39 cm ID). Recoveries for MTX and DAMPA were determined using radiolabelled standards. The reported recoveries were 77%, 79% for MTX and DAMPA from urine and 90% and 89% for MTX and DAMPA from plasma. They did not report a figure for the recovery of 7-OHMTX.

Howell et al (1980) have separated MTX, 7-OHMTX and DAMPA from human plasma. Polyglutamates were not studied since they were obscured by endogenous plasma components. The sensitivity of this method was 2×10^{-7}M for MTX, 7-OHMTX and DAMPA and the quoted precision was a coefficient of variation (CV) of 10-20%.

Few of these assays incorporated an internal standard. The extraction procedures employed gave a wide range of recovery figures particularly for the metabolites for which the assays were not optimised.

None of the authors mentioned above reported the investigation of possible interference by other anti-neoplastic drugs. This is surprising in view of the common use of more than one drug in current chemotherapeutic regimes.

AIMS

The aims of this study were:-

(1) To develop a sensitive, accurate, precise method for MTX and its metabolites in biological fluids.

(2) To study the pharmacokinetics in patients treated with high and low doses of MTX and examine the clinical relevance of MTX assay and measurement of metabolites to the safe use of MTX in vivo.

CHAPTER 3

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF METHOTREXATE AND ITS METABOLITIES

INTRODUCTION

The several high performance liquid chromatography (HPLC) methods used to achieve specificity for MTX by chromatographic separation of the drug from its metabolites, and published before the beginning of this study, are reviewed here (for more details see Chapter 2).

All extraction procedures involved precipitation of the proteins by trichloroacetic acid or perchloric acid. In some, the supernatant was injected directly onto the HPLC column (Nelson et al, 1977; Lankelma et al, 1978). In others the protein-free plasma was extracted directly with organic solvent (Howell et al, 1980). Watson et al (1978) used an organic solvent extraction following ammonium sulphate addition. Adsorption extractions were carried out using anion-exchange resin (Donehower et al, 1979).

Only Nelson et al (1977) and Watson et al (1978) used an internal standard in order to improve precision.

Reverse-phase chromatography was used by some authors (Nelson et al, 1977 , Lankelma et al, 1978; Donehower et al, 1979 and Howell et al, 1980) and others used anion-exchange chromatography (Lankelma et al, 1978; Watson et al, 1978). Most authors used an ultraviolet (UV) detector while Nelson et al (1977) found that the sensitivity for MTX could be increased about 40-fold (2×10^{-8}M) when a fluorescence detector was used.

The major metabolite, 7-OHMTX was detected in plasma by Watson et al (1978) and Howell et al (1980) and in urine by Donehower et al (1979).

DAMPA was reported in urine by Donehower et al (1979) and in serum by Howell et al(1980). Polyglutamates have been chromatographed from standard solutions (Howell et al, 1980) but not from plasma because of failure to separate these from endogenous components.

Not all authors have quoted figures for the recovery, precision, sensitivity, accuracy and linearity of their methods, hence the methods cannot be compared directly. Surprisingly none of the authors quoted checked for possible interference by other drugs.

A resume of the performance characteristics of published HPLC methods is presented in Table 4.

Ion-Pair Chromatography

Many organic molecules contain highly polar groups which make them difficult to chromatograph satisfactorily using polarity separation. They may however be retained by using ionised surfactants, these lipophilic ions adsorb to reverse-phase packings, ions of opposite charge form ionic bonds with them, thereby causing retention. For example, the sulphonic acids are ionised throughout the pH range and are useful in the analysis of cationic compounds (Knox et al, 1977). An ion-pair may be described as shown in the equation:

TABLE 4 The Characteristics of Published HPLC Methods for MTX and its Metabolites

	Recovery	Precision	Sensitivity	Analogues Studied	Linearity
Nelson et al, 1977 UV Detector	40%	-	8×10^{-7}	Folic Acid Leucovorin	-
Nelson et al, 1977 Fluorescence Detector	-	10%	2×10^{-8}	Folic Acid Leucovorin	2×10^{-8}M - 2×10^{-5}M
Lankelma et al, 1978	70.2%	5% and 2% (1)	2×10^{-8}	-	-
Watson et al, 1978	26% (7-OHMTX) (2)	3%	1×10^{-7}	-	2×10^{-7}M - 2×10^{-5}M
Donehower et al, 1979	77% u, 90% s MTX (3) 79% u, 89% s DAMPA	-	-	-	-
Howell et al, 1980	-	10-20%	2×10^{-7}	-	-

(1) The precision was 5% and 2% for concentrations of 4×10^{-8} and 8×10^{-8} respectively.

(2) No recovery was quoted for MTX.

(3) The abbreviation u and s indicate urine and serum respectively.

$$S^-aq + P^+aq \rightleftharpoons S^-P^+org$$

Where S^- is solute ion, P^+ is pairing ion, and SP is solute ion-pair. The extraction coefficient (E_{SP}) of the ion-pair is:

$$E_{SP} = [SP_{org}]/[S^-a_q][P^+a_q]$$

The distribution coefficient (D_S) of the solute is defined by:

$$D_S = [SP_{org}]/[S^-a_q] = [E_{SP}][P^+a_q]$$

Definition: $$k' = \frac{\text{retention volume of analyte - to}}{\text{retention volume unretained peak (to)}}$$

Since k' is proportional to D_S (distribution of the solute) in reversed-phase chromatography, k' will be proportional to the pairing ion concentration, i.e. k' increases with increasing ion-pair concentration and also with increasing chain length of the ion-pairing agent.

MATERIALS AND EQUIPMENT

Materials

MTX, DAMPA and calcium leucovorin were donated by Lederle (Gosport, Hants, England). MTX diglutamate and MTX triglutamate were made available by Professor C M Baugh, University of South Alabama, USA. 7-OHMTX was a gift from Dr David G Johns (National Cancer Institute, Bethesda, Maryland, USA). 7-OHDAMPA was synthesised in

vitro and the identity confirmed by electron impact mass spectrometry and nuclear magnetic resonance spectroscopy (Chapter 4). L-tryptophyl L-glutamic acid, used as internal standard, was obtained from the Sigma Chemical Company (Dorset, England). Hexanesulphonic acid was Fisons HPLC grade (Fisons Scientific Apparatus, Longborough, England). The anion-exchange resin Dowex 1-x2 was obtained from Bio-Rad Laboratories (Watford, Hertfordshire, England). Acetonitrile, HPLC grade was obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland). Methanol was obtained from James Burroughs Limited (London, England) and all other reagents were from British Drug Houses (Pool, Dorset, England). Polypropylene Econo-columns (0.7 cm ID x 4 cm) with a 10 ml reservoir and porous 70 u polyethylene bed support were obtained from Bio-Rad (Watford, Hertfordshire, England).

Choice of L-Tryptophyl L-Glutamic Acid as Internal Standard (IS)

A variety of substances were investigated in order to find a suitable internal standard (IS). The only simple readily available compound which was both soluble and had an acceptable k' was p-nitrophenol. However an internal standard should be structurally similar to the analyte and exhibit similar physiochemical properties; p-nitrophenol is unstable and was more readily extracted from aqueous solutions than MTX. L-tryptophyl L-glutamic acid was a more satisfactory alternative as it had a

structure somewhat analogous to MTX, was available commercially, was found to have similar physiochemical properties to MTX in the analytical system and was resolved from MTX and its metabolites on the HPLC column.

Apparatus

A Pye-Unicam HPLC system (Cambridge, England) consisting of an LC-XPS single piston reciprocating pump, a LC-UV variable wavelength detector and a PM8521 single pen recorder were used.

The stainless steel column (10 cm x 5 mm ID) was obtained from Shandon Southern (Runcorn, England) slurry packed with 5 u ODS Hypersil (Shandon Southern) using a Shandon slurry packer and fitted with a valve adaptor (Shandon Southern). An 8050 Autosampler (Varian Associates, Walton-on-Thames, England), with a 20 ul loop was used for urine injection according to the manufacturers recommendation. Because of the limited amount of analyte available from serum extracts the operation of the autosampler was modified in order to reduce the dilution factor.

The autosampler used gas pressure to force the liquid through the sample line to the loop in the automatic valve injector. The volume of sample used is dependent on the dead volume of the sample line/injector and the gas pressure applied. By fitting a small gas pressure gauge and adjusting the outlet pressure to 50 N/m^2 it was possible to inject 20 ul of sample from a

100 ul aliquot using approximately 75 ul of the aliquot; manual injection when required was made via a Rheodyne 7125 injection valve (Scotlab Instruments Sales Limited, Law, by Carluke, Scotland).

The data generated was handled by an Infotronic CRS 304-40 integrator (Belmont Instruments Limited, Glasgow, Scotland).

METHODS

Initial Chromatography

A reverse-phase material (ODS Hypersil) was used to separate MTX and its metabolites. The metabolites consisted of MTX diglutamate($MTXG_1$) MTX triglutamate ($MTXG_2$) 7-OHMTX, and DAMPA with leucovorin and L-tryptophyl L-glutamic acid (IS). The liquid chromatographic method was developed as follows:

1. Polarity investigation

Methanol was first used alone as a mobile-phase, to elute the MTX and its metabolites. Increases in polarity were effected by adding water to the methanol. The changes in k' of MTX and its metabolites were studied.

2. pH control

The pH of the mobile-phase was controlled by using 0.05 M phosphoric acid (pH 2.2). Methanol:0.05 M phosphoric acid (50:50, v:v.) was used initially. The effect of changes in polarity at this pH was examined.

3. Ion-Pairs

Hexanesulphonic acid was used as ion-pair at a concentration of 5.3 mM in 0.05 M phosphoric acid.

Sample Preparation

1. Solvent selection

Two ml of an aqueous solution containing 8.8 uM MTX and 12 uM DAMPA standard was shaken in a C14/C15 Q + Q tube with 5 ml of one of the following solvents; ethyl acetate:isopropanol (10:1), dichloromethane, hexane, diethyl ether, cyclohexane and hexane containing 0.5-5% v/v amyl alcohol.

The contents of the tubes were shaken for 10 minutes, and centrifuged for 10 minutes at 2000 g, 4 ml of the organic layer was transferred to a C14/C15 conical quick fit tube and the organic solvent evaporated to dryness at 50°C under a stream of nitrogen The residue was dissolved in 100 ul of mobile-phase and 20 ul was injected onto the HPLC column. The recovery was calculated by external standarisation.

2. Solvent volume selection

Selection of the appropriate volume of ethyl acetate:2-propanol (10:1) was performed as in the experiments for solvent selection but with different volumes of ethyl acetate:2-propanol ranging from 1-10 ml.

3. Protein precipitation

Different volumes of precipitant (the volumes depending on the type) were added to 1 ml human serum containing 10 ug MTX standard (10 ug MTX/ml is equivalent to 2 x 10^{-5}M), mixed and centrifuged for 10 minutes at 2000 g. The supernatant was removed and extracted with the appropriate volume of ethyl acetate:2-propanol. The organic layer was evaporated to dryness under a stream of nitrogen in a water bath at 50°C. The residue was dissolved in 100 ul mobile-phase and 20 ul was injected onto the HPLC column. The methods of protein precipitation used were as follows:

(a) Perchloric acid

One ml of 0.69 M perchloric acid was added to 1 ml of serum, the sample spun and the supernatant removed. The pH of the supernatant was neutralised using 1M potassium hydroxide solution, and extracted with 5 ml of ethyl acetate:2-propanol (10:1). The use of Molar potassium carbonate instead of potassium hydroxide was also studied. A further sample was extracted without neutralisation of the perchloric acid.

(b) Trichloroacetic acid (TCA)

0.3 ml of 0.53M TCA was added to 1 ml of serum. Following centrifugation, the supernatant solution was extracted directly with 5 ml of ethyl acetate:2 propanol (10:1).

(c) Copper tungstate

One ml of 0.4M sodium tungstate and 1 ml of 0.6M copper sulphate solution was added to 2 ml serum. This method yielded a heavy precipitate, the analysis was completed as in (b).

(d) Tungstic acid

0.3 ml 0.4M sodium tungstate and 0.3 ml 0.3M sulphuric acid was added to 1 ml of serum. The supernatant was extracted as in (b).

(e) Sulphosalicylic acid

One ml of 0.4M sulphosalicylic acid was used to precipitate the protein in 1 ml of serum. The analysis was completed as in (b).

(f) Heat precipitation

0.8 ml of 1M phosphate buffer pH 6.2 was mixed with 2 ml serum, and a volume of water ranging from 0.5-4 ml was added to ascertain the volume of water which gave the highest recovery. The tubes were covered and heated in a boiling water bath for 3 minutes. After cooling, they were centrifuged at 2000 g and 20 ul of the supernatant solutions were injected on to the HPLC column.

4. Extraction from protein free supernatant solution

The efficiency of solvent extraction from this protein-free supernatant solution was investigated. The following modifications to the heat precipitation method were performed:-

(a) Hexanesulphonic acid

Addition of hexanesulphonic acid to the supernatant in the range of 2.6-53 mM was tried.

(b) pH of the supernatant

The effect of altering pH of the supernatant was tested over the range from 2-8, by addition of either 0.1M phosphoric acid or 0.1M sodium hydroxide.

(c) Addition of solid ammonium sulphate (Watson et al, 1978)

Following adjustment to pH4 with 0.1M phosphoric acid, 5 gm solid ammonium sulphate was mixed with 2 ml of the protein-free solution, the mixture was extracted with ethyl acetate:isopropanol (10:1 v:v).

(d) Activated charcoal

The supernatant was shaken with activated charcoal which was removed by filtering through micropore filters (0.22 u) (Millipore Corporation, Bedford).

The charcoal was washed with water, and the drug was eluted using a mixture of ethanol containing 0.28M ammonium hydroxide and was evaporated to dryness. The residue was washed in 100 ul mobile-phase and 20 ul was injected on to the HPLC column.

(e) Acetonitrile

Acetonitrile was used instead of ethyl acetate: isopropanol as extraction solvent in order to reduce contamination.

(f) Liquid solid extraction (Donehower et al,1979)

The supernatant was applied to a column of Dowex 1-X2 anion-exchange resin, and 10 ml of distilled water was used to wash the column. MTX was then eluted using 4 ml 2M acetic acid. The acetic acid was evaporated to dryness, the residue was dissolved in mobile-phase and 20 ul was injected onto the HPLC.

The above method was modified as follows:-

(i) Supernatant and serum comparison

A comparison was carried out between applying the supernatant following protein precipitation to a Dowex 1-X2 column and applying the serum directly.

(ii) pH of the sample

The ionisation of MTX in plasma was increased by raising the initial pH to 8.0 and the effect on recovery from the column was determined.

(iii) Optimisation of column size

Columns ranging in height from 0.5 cm to 4 cm were studied in order to select the most appropriate column bed dimension.

(iv) Comparison of deionised and distilled water

Columns were washed either with 10 ml deionised or distilled water after loading with serum containing MTX and DAMPA. The water

portions were evaporated to dryness, the residue was dissolved in 100 ul mobile-phase and 20 ul was injected on to the column.

(v) Methanol

Following washing with water a further wash with 4 ml methanol was added and the methanol analysed by HPLC after evaporation to a 100 ul volume.

(vi) Elution with acetic acid/methanol

In order to determine the optimum concentration for elution of MTX and its metabolites an experiment was carried out using different concentrations of acetic acid in methanol over the range of 5-40%.

(vii) Step-wise elution using two sequential elution steps

An experiment was carried out using 2 ml of 5% acetic acid in methanol followed by 4 ml of 25% acetic acid in methanol as eluant. This procedure was compared with a single elution with 4 ml of 25% acetic acid.

Quantitation

The concentrations of MTX and metabolites in patients sera were calculated by ratio of the peak area of MTX (or one of its metabolites) to the peak area of the internal standard and comparison with the standard curve. The urine concentrations were determined by using an external standardisation procedure as previously

described (Watson et al, 1980). The target standard was 1.1×10^{-4}M (50 mg/l), which was injected after every fourth sample. The values obtained from this "correction" standard were referred to the value for the 1.1×10^{-4}M standard used in the calibration line. Any variation in drift standard was corrected and the correction was applied to the two unknown samples on either side of that standard.

Linearity and Sensitivity

The linearity was determined as a function of on-column sample weight by chromatographing 20 ul aliquots of aqueous solutions of MTX, 7-OHMTX and DAMPA over a concentration range of 0-500 mg/l (1.1×10^{-3}M) (equivalent to 0-10 ug on column weight).

Recovery

Solutions of MTX, 7-OHMTX and DAMPA in serum covering the range 0-50 mg/l (1.1×10^{-4}M) were analysed, the difference in detector response between the extracted and directly injected aqueous standard represented the absolute percentage recovery. Sera of known MTX concentrations to which internal standard had been added were assayed and the results compared with calibrators to represent relative recovery.

Precision

1. Serum

The precision was assessed by determining MTX in 16 serum extracts at each of 3 different concentrations, 4.4×10^{-7}M, 2.2×10^{-5}M and 1.16×10^{-4}M (0.2 mg/l, 10 mg/l and 53 mg/l).

2. Urine

Thirty-four direct injections from one patient's urine sample containing MTX, 7-OHMTX, DAMPA and 7-OHDAMPA were made using the autosampler and the results compared. No internal standard was used.

Accuracy and Specificity

1. Serum and urine

The accuracy was determined from recovery studies. The possibility of interference by a number of drugs, especially anti-cancer drugs, was checked by the addition of aqueous solutions of these to the same pooled serum as was used for the preparation of MTX solutions. The drugs studied were; cyclophosphamide, dacarbazine, fluorouracil vincristine, daunorubicin, doxorubicin, cis-platinum, cytarabine, trimethoprim, paracetamol and diazepam. The last two were added because of the frequency with which they were co-prescribed with MTX in patients under investigation in this hospital, and trimethoprim in view of the reported interference caused by this drug with other methods for determining MTX (Hande , Gober and Fletcher, 1980).

2. Correlation with homogenous immunoassay

Sera from 50 patients were analysed by immunoassay (EMIT) as well as by the HPLC method. The correlation was plotted.

RESULTS

Initial Chromatography Method

1. Polarity investigation

The capacity ratio (k') for MTX and its metabolites when methanol was used alone as mobile-phase was found to be zero. Increasing the polarity by adding water to 80% v/v failed to increase the retention of MTX or any of its metabolites except DAMPA which had k'of 1.

2. pH control

Using an aqueous component at pH 2.2 the k' for leucovorin (L), L-tryptophyl L-glutamic acid (IS), MTX triglutamate (G_2), MTX diglutamate (G_1), methotrexate (MTX), 7-hydroxymethotrexate (7-OHMTX) and 4-amino N^{10}-methylpteroic acid (DAMPA) were 0.16, 0.41, 0.58, 0.58, 0.75, 0.75 and 2.25 respectively.

Table 5 shows the effect of increasing polarity at pH 2.2 on the reduced plate height (h value), capacity ratios (k') and resolution factor (Rs) for MTX and its metabolites.

3. Ion-pair

The elution pattern obtained using the mobile-phase with and without hexanesulphonic acid as ion-pair is shown in Table 6, from which it can be seen that the resolution and efficiency were improved.

An acceptable mobile-phase composition for separation of MTX and its metabolites with internal standard was found to be methanol:0.05M phosphoric acid

TABLE 5

Comparison of k', Rs and Efficiency obtained with Solvents containing Different Proportions of MEOH: 0.05M H_3PO_4

	LEUCOVORIN		IS		G_2		G_1		MTX		7-OHMTX		DAMPA
SOLVENT	50 : 50	MEOH : 0.05M H_3PO_4											
Reduced plate height	102		69		55		55		45		70		50
k'	0.16		0.41		0.58		0.58		0.75		0.75		2.25
Rs		0.75		0.5		0		0.5		0		2.3	
SOLVENT	40 : 60	MEOH : 0.05M H_3PO_4											
Reduced plate height	69		54		70		32		18		25		37
k'	0.41		1		1.08		1.1		1.75		1.91		4.75
Rs		1.5		0.22		0.12		1.16		0.44		4	
SOLVENT	30 : 70	MEOH : 0.05M H_3PO_4											
Reduced plate height	50		69		66		96		33		24		60
k'	0.66		1.8		3.3		3.5		4.58		5.5		11.1
Rs		2.3		1.8		0.16		1		1		3.16	

TABLE 6

Comparison between solvents with and without Ion-Pair Agents

	Solvent A			Solvent B		
	k'	Rs	h	k'	Rs	h
Leucovorin	0.66		50	1.23		25
		2.3			7.6	
Internal STD	1.8		69	4.46		8.9
		1.8			1.38	
G_2	3.3		66	5.15		9.5
		0.16			1.01	
G_1	3.5		96	5.6		10.0
		1			1.5	
MTX	4.58		33	6.6		8.16
		1			2.58	
7-OHMTX	5.5		24	8.3		6.9
		3.16			8.8	
DAMPA	11.1		60	18.1		8

Solvent A: Methanol:0.05M phosphoric acid (pH 2.2) 30:70

Solvent B: Methanol:0.05M phosphoric acid (containing 5.3 mM hexanesulphonic acid) 30:70

pH 2.2 containing 5.3 mM hexanesulphonic acid in proportions of 30:70 v:v. Subsequent experience indicated that minor adjustment to this eluent were needed to maintain an optimum separation.

The chromatogram of a standard injection of MTX and its metabolites with L-tryptophyl L-glutamic acid as internal standard using as mobile-phase methanol:0.05M H_3PO_4 containing 5.3 mM hexane sulphonic acid (28:72) is shown in Figure 6.

Optimised Liquid Chromatography Method

The mobile-phase consisted of 5.3 mM hexanesulphonic acid in 50 mmol/l phosphoric acid:methanol (72:28), the operating conditions were: temperature ambient; flow rate 1 ml/minute; wavelength 307 nm; chart speed 0.5 cm/minute. Absorbance settings varied depending upon the samples injected.

Sample Preparation

1. Solvent selection

Ethyl acetate:isopropanol in proportions of 10:1 were found to produce the highest recovery for MTX and DAMPA (Table 7).

2. Solvent volume selection

The best volume was found to be 5 ml of ethyl acetate:isopropanol in proportions of 10:1 to extract 2 ml of aqueous standard (Table 8).

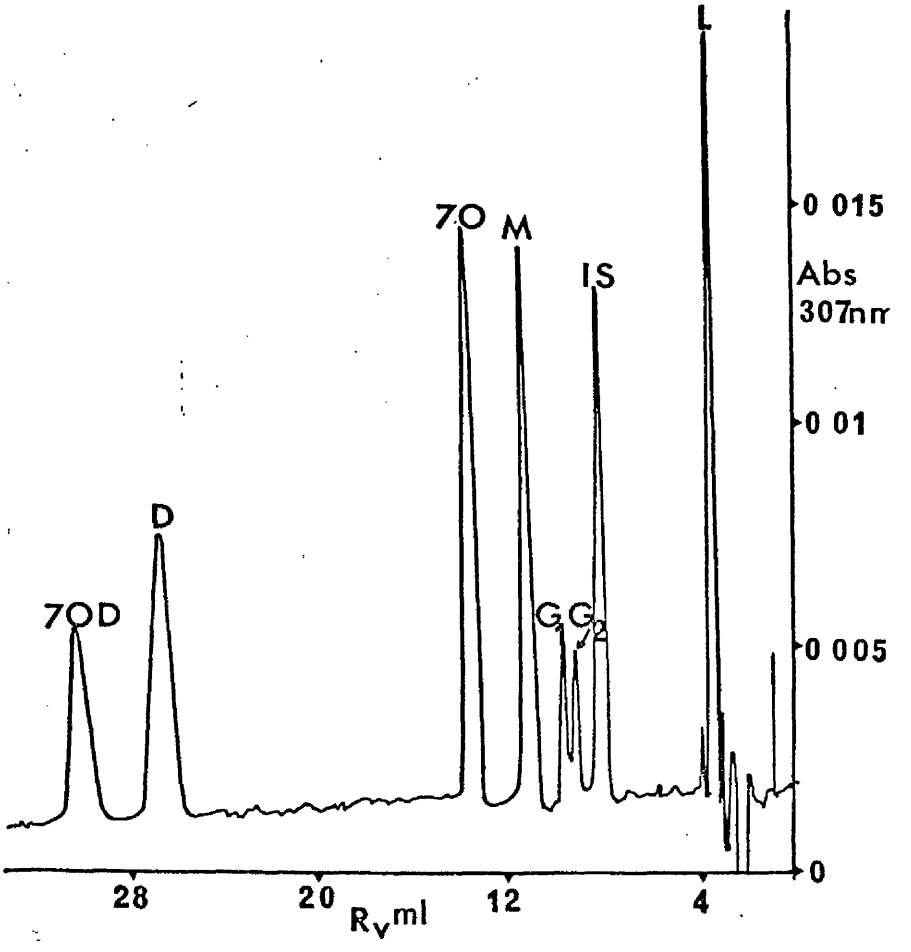


FIGURE 6 Chromatogram of MTX and metabolite standards with L-tryptophyl L-glutamic acid as internal standard, peak identities are:

1. Leucovorine (L)
2. L-tryptophyl L-glutamic acid (IS)
3. MTX triglutamate (G_2)
4. MTX diglutamate (G_1)
5. MTX (M)
6. 7-OHMTX (7O)
7. DAMPA (D)
8. 7-OHDAMPA (7OD)

TABLE 7

Absolute Recoveries of MTX and DAMPA following Extraction from Aqueous Solution by Different Organic Solvents (n = 4)

Organic Solvent	Recovery of MTX	Recovery of DAMPA
Hexane	0	3.9%
Hexane + amyl alcohol (the alcohol in concentration ranging from 0.5-5% v/v)	0	0
Cyclohexane	0	6.3%
Diethyl ether	0	38.8%
Dichloromethane	10%	13%
Ethyl acetate:2-propanol (10:1)	78.6%	92%

TABLE 8

Absolute Recoveries of MTX and DAMPA following Extraction by Different Volumes of Ethyl Acetate: 2-Propanol (10:1) (n = 4)

ml of Ethyl Acetate: 2-Propanol (10:1)	Recovery of MTX	Recovery of DAMPA
1	30%	20%
2	30%	22%
3	53%	42%
4	63%	65%
5	80%	90%
6	76%	85%
7	77%	86%

3. Protein precipitation

(a) Perchloric acid

The absolute recovery was low (< 30%) using either 1M potassium hydroxide , 1M potassium carbonate or no neutralisation.

(b) Trichloroacetic acid

The absolute recovery was low (< 30%).

(c) Copper tungstate

No MTX was recovered.

(d) Tungstic acid

Recovery of MTX was zero.

(e) Sulphosalicylic acid

Low absolute recovery (< 35%) was obtained.

(f) Heat Precipitation

The effect of the volume of water on the absolute recovery after heat precipitation is shown in Table 9.

4. Extraction from protein-free supernatant solution

Direct extraction of the supernatant gave 14% recovery. The following were modifications to the heat precipitation method.

(a) Hexanesulphonic acid

Addition of hexanesulphonic acid to the supernatant and extraction with ethyl acetate:isopropanol in ratio of 10:1 gave no increase in absolute recovery (14%).

(b) pH of the supernatant

It was found that pH 4 yielded the highest absolute recovery but this was still less than 40% (Table 10).

TABLE 9

The Effect on the Recovery of MTX of Dilution of Serum before Heat Precipitation

Serum	Phosphate Buffer pH 6.2	Water	Recovery
2 ml	Nil	Nil	Incomplete precipitation
2 ml	0.8 ml	0.5	Incomplete precipitation
2 ml	0.8 ml	1.0	75%
2 ml	0.8 ml	1.5	85%
2 ml	0.8 ml	2	95%
2 ml	0.8 ml	2.5	90%
2 ml	0.8 ml	3	93%

TABLE 10

The Effect of pH on the Extraction of MTX from a Protein Free Supernatant by Ethyl Acetate:Isopropanol

pH	Recovery of MTX
2	10%
2.5	12%
3	14%
4	39%
5	30%
5.5	21%
6	14%
7	14%
8	10%

(c) Addition of solid ammonium sulphate

This procedure gave an absolute recovery of 74%. However the procedure gave rise to many peaks from blank sera which would interfere with the peaks obtained for MTX and its metabolites.

(d) Activated charcoal

The absolute recovery was 3.6%.

(e) Acetonitrile

The absolute recovery was 75% but interfering peaks were not found in extracts from drug-free serum

(f) Liquid solid extraction

This procedure showed a good absolute recovery (77%) and clean chromatograms for drug-free sera were obtained, but the use of water as eluant resulted in lengthy evaporation times.

(i) Supernatant and serum comparison

Better recovery was obtained when serum was applied directly (Table 11). This method was preferred since it showed the highest recovery as well as being the least time consuming.

(ii) pH of the sample

Raising the pH to 8.0 gave an increase in recovery of 7-OHMTX from 66% to 74%. MTX remained at 90%.

TABLE 11

Recovery of MTX and Metabolites from Dowex 1-X2 Column before and after heat precipitation

	Recovery of MTX	Recovery of 7-OHMTX	Recovery of DAMPA
Application of supernatant after heat precipitation	67%	49%	73%
Direct application of serum	90%	66%	85%

(iii) Optimisation of column size

It was found that a 2 cm column was optimal for 1 ml of serum. Below 1 cm column capacity was insufficient. Above 2 cm k' was increased.

(iv) Comparison of deionised and distilled water

A proportion of the MTX was eluted by distilled water, presumably due to its acidity (pH 5). Deionised water at neutral pH caused no elution.

(v) Methanol

Following washing by methanol very clean blanks were obtained.

(vi) Elution with acetic acid:methanol

It was found that a solution of 25% acetic acid in methanol was the lowest concentration which yielded an acceptable recovery of MTX (Table 12). This eluant is an azeotropic mixture and consequently improves the rapidity of the evaporation.

(vii) Stepwise elution

It was found that the two-step procedure gave a further increase in recovery resulting in 95% retrieval of added MTX from serum.

TABLE 12

Effect of Acetic Acid on the elution by Methanol of MTX and DAMPA from Dowex 1-X2

Percentage of acetic acid in methanol composition	Recovery of MTX	Recovery of DAMPA
0%	0%	0%
5%	65%	60%
10%	81%	87%
15%	84%	87%
20%	90%	89%
25%	96%	94%
30%	94%	94%
35%	95%	90%
40%	96%	94%

Optimised Extraction Procedure

1. Serum

One hundred ul internal standard (as per Table 13) was added to 1 ml serum, the pH was adjusted to 8 with 50 ul 0.2M potassium carbonate solution and the sample applied to an ion-exchange column prepared by packing a Pasteur pipette with a 2 cm column of Dowex 1-X2. The column was first washed with 10 ml deionised water followed by 4 ml methanol. MTX and its metabolites were eluted using 2 ml of 5% acetic acid in methanol, followed by 4 ml of 25% acetic acid in methanol. The acid fractions were combined and evaporated to dryness under vacuum at 35°C. The residue was dissolved in 100 ul of HPLC mobile-phase, vortexed for 15 seconds, centrifuged for 1 minute and 20 ul injected on the column via the autosampler.

For low concentrations of MTX the residue was dissolved in 25 ul HPLC mobile-phase and 20 ul of this injected manually on the column.

2. Urine

Direct injection of urine (20 ul) yielded satisfactory chromatography of MTX and its metabolites.

TABLE 13

Internal Standard Concentration and Detector Attenuation used at different MTX Concentrations

MTX Concentration Range mg/l	M	Stock IS g/l	Detector AUFS
0-0.09	$0 - 2 \times 10^{-7}$	0.25	0.04
0.09-0.9	$2 \times 10^{-7} - 2 \times 10^{-6}$	1	0.04
0.9 up	2×10^{-6} - up	5	0.16

Linearity and Sensitivity

1. Serum and Urine

The assay for MTX was linear over the range 0-10 ng on column sample weight for extraction from serum with internal standard or for directly injected aqueous standard, equivalent to 0-50 mg/l (0-1.1 x 10^{-4}M) and 0-500 mg/l (0-1.1 x 10^{-2}M) respectively.

The lowest detectable serum concentration (defined as a signal to noise ratio of 2) was 12.5 ug/l (2.75 x 10^{-8}M) and for urine MTX 500 ug/l (1.1 x 10^{-6}M), equivalent to 10 ng on column.

Recovery

Absolute recoveries of MTX, 7-OHMTX and DAMPA from serum are detailed in Table 14. Recoveries relative to the L-tryptophyl L-glutamic acid internal standard, as calculated by the ratio of peak areas, was found to be 96.1$\pm$3.8 (n = 16) for MTX. As urine is injected directly on column, there was no need to determine recovery.

Precision

The precision studies are summarised in Table 15. All except 7-OHDAMPA fell below 6% CV.

Accuracy and Specificity

1. Serum and urine

None of the drugs examined interfered with the assay of MTX and its metabolites. Over 200 MTX-free sera have been assayed with no interference. Although no

TABLE 14

Absolute Recovery of MTX, 7-OHMTX and DAMPA from Serum (n = 16)

Compound	Absolute Recovery (%)		
	$\bar{x}$	±SD	CV%
MTX	94.5	4.8	5
7-OHMTX	72.4	5.8	8.1
DAMPA	89.5	5.8	6.4

TABLE 15

Precision Studies on MTX in Serum (n = 16) and MTX and Metabolites in urine (n = 36)

Compound	Matrix	Concentration			CV%
		Mass Units		Molar Units	
MTX	Serum	162	ug/l	3.56×10^{-7} M	4.2
MTX	Serum	9.6	mg/l	2.1×10^{-5} M	4.1
MTX	Serum	50	mg/l	1.1×10^{-4} M	5.1
MTX	Urine	42.4	mg/l	9.3×10^{-5} M	2.6
7-OHMTX	Urine	15.8	mg/l	3.35×10^{-5} M	5.6
DAMPA	Urine	71.1	mg/l	21.0×10^{-5} M	2.7
7-OHDAMPA	Urine	6.68	mg/l	1.98×10^{-5} M	6.4

extraction was used for urine samples, no interference was found here either. Over 200 MTX-free urines have been analysed and no interference noted.

2. Correlation with homogenous immunoassay

The correlation between the HPLC method and the homogenous immunoassay was found to be adequate for samples during the first 48 hours (Figure 7) but it might be effected by the presence of 7-OHMTX in the later stages or following low-dose therapy.

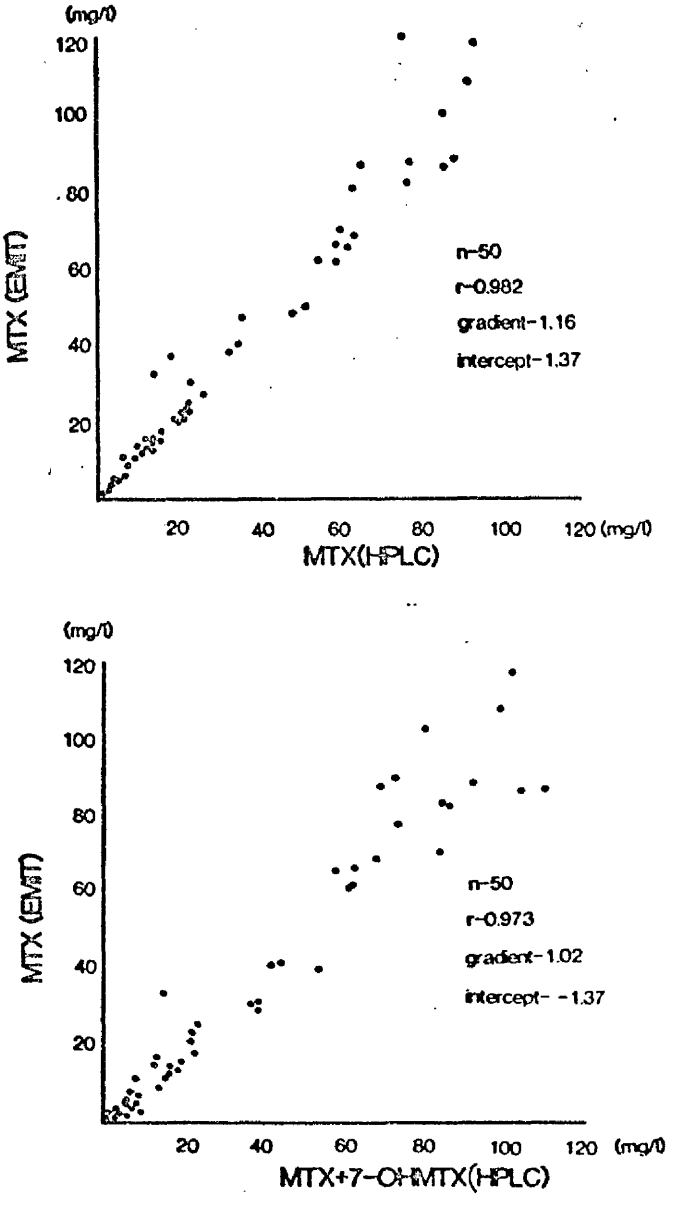


FIGURE 7 Association between serum MTX concentrations measured by EMIT and:

a) Serum MTX measured by HPLC.

b) The sum of serum MTX and 7-OHMTX measured by HPLC.

DISCUSSION

In the initial studies, it was found that changing the polarity did not improve the chromatography of MTX and its metabolites. Adjustment of pH to 2.2 gave increased retention (Table 5). The values which it was aimed to achieve were: reduced plate height < 10, k' between 1 and 11 and resolution > 1.25.

Efficiency did not correlate with polarity, but with increasing polarity, leucovorin and DAMPA approached an acceptable k' value. Resolution improved as retention volume increased, however the resolution between MTX and 7-OHMTX and between $MTXG_2$ and $MTXG_1$ were insufficient even at high k'. The addition of an ion-pairing agent gave further improvements in efficiency in that every component, except for leucovorin, had h = < 10 and k' increased (leucovorin from 0.66 to 1.23 and DAMPA from 11.1 to 18.1) which were acceptable because baseline resolution was achieved between all components except G_2 and G_1. Since these are expected to be present at approximately equal concentrations a resolution of 1 was accepted. Minor adjustments in the methanol content of the mobile-phase were subsequently required to maintain optimal resolution.

The wavelength used for detection of MTX and its metabolites was 307 nm since this was both optimal for the majority of analytes and clear of much potential endogenous interference. In the initial studies,

ethyl acetate:isopropanol (10:1) was selected as the appropriate extracting solvent for plasma due to the high recoveries obtained from aqueous standards. It is highly polar, with a Hildebrand solubility parameter of 0.66. A minimum volume ratio of 2.5:1 of solvent to aqueous-phase provided maximum extraction which was still, however, only 80% for MTX. At this stage no pH adjustment was considered.

All of the protein precipitants gave rise to lower recoveries due to adsorption of MTX on precipitated plasma proteins (Chakrabarti and Bernstein, 1969). Copper tungstate and tungstic acid in particular produced a heavy precipitate in which MTX was presumably trapped, since no MTX was found in the supernatant when these compounds were used. It is of interest that other authors claim relatively high recoveries after protein precipitation (Watson et al, 1978; Howell et al, 1980). Their studies did not employ internal standards to control the extraction stage.

Heat precipitation with direct injection of the supernatant onto HPLC column yielded a relatively good recovery which could be further improved by dilution of serum with water. Although recoveries were of the order of 85%, sensitivity was low due to the dilution of the original plasma volume. It was therefore decided to extract this protein-free supernatant with the solvent

system optimised as above. However the maximum absolute recovery using the extraction of protein-free supernatant was only 40%.

Direct application of serum to a Dowex 1-X2 column gave both higher recovery and higher sensitivity than application of protein-free supernatant to such a column due to the absence of dilution.

This liquid solid extraction had been used successfully by Donehower et al (1979) who did not record the absolute recovery of 7-OHMTX. In the current study, adjustment of the serum to pH 8 was found to be necessary to ensure that MTX and its metabolites would effectively interact with the ion-exchange resin; failure to perform this step led to reduced recoveries of 7-OHMTX. The methanol wash yielded cleaner blanks than the water wash employed by Donehower et al. Acidity is required to elute MTX and its metabolites, methanol/acetic acid was used since they form an azeotropic mixture with a consequent improvement in the rapidity of the evaporation step.

Any HPLC method which requires an extraction step is slower than a one-step assay such as an optical immunoassay. However, for pharmacokinetic studies it is necessary to measure the unchanged drug and also its major metabolites in plasma and urine. It has been

reported that 7-OHMTX cross-reacts with the immunoassays by about 10% and this was borne out with the associations reported here.

Since the 7-OHMTX concentration may be higher than MTX concentration at the time at which toxicity is assessed i.e. 24 or 48 hours, this is a potential source of error.

The accuracy and sensitivity of the HPLC assay make the use of this technique preferable to the immunoassay procedure at low concentrations. The immunoassay procedure may be preferred where rapid result turnround is required during high-dose MTX therapy.

The HPLC procedure described here is highly sensitive and linear over a wide range and compares well with optical immunoassay which is an order of magnitude less sensitive and linear over only 5% of the range covered by the HPLC procedure (Farid, Watson and Stewart, 1983). Recently HPLC methods were developed by Lawson, Dixon and Aherne (1981); Breithaupt, Kuenzlen and Goebel (1982) and Collier, MacLeod and Soldin (1982) which are less sensitive than the procedure described here. Chen and Chiou (1981) have since produced a cationic-exchange chromatography method which has the same sensitivity for MTX as this method.

Experience in this laboratory indicates that in order to produce acceptable between-batch precision, all extraction procedures should employ an internal standard.

Many workers quoted above did not use an internal standard. The internal standard L-tryptophyl L-glutamic acid, exhibited similar physiochemical behaviour to MTX in the analytical system and was ideally resolved from MTX and its metabolites on the HPLC column.

Other workers (Nelson et al, 1977; Watson et al, 1978 and Lawson et al, 1981) used an internal standard which was not commercially available. The L-tryptophyl L-glutamic acid, used in these experiments is commercially available. The method presented here gives a simple reliable extraction procedure for the rapid determination of MTX, 7-OHMTX, DAMPA and 7-OHDAMPA in serum and urine by HPLC. This method could also detect the MTX diglutamate and MTX triglutamate.

So far as is known, this method is the only one which can be used to separate MTX and all metabolites (7-OHMTX, DAMPA, 7-OHDAMPA, MTX diglutamate and MTX triglutamate) with an internal standard in one chromatogram. It is therefore suitable for studying the intracellular metabolites of MTX e.g. in erythrocytes. The procedure is sufficiently sensitive to detect MTX following administration of low doses used in e.g. psoriasis and is to be preferred if low MTX concentrations are to be determined.

CHAPTER 4

IN VITRO SYNTHESIS OF METHOTREXATE METABOLITES

INTRODUCTION

7-OHMTX, present in plasma is thought to be the product, not of the cytochrome P450 system but of hepatic aldehyde oxidase. Aldehyde oxidase was first isolated from pig liver (Gordon, Green and Subrahmanyan, 1940) and identified as a flavoprotein which could catalyse the oxidation of a variety of aliphatic and aromatic aldehydes. In 1946, Knox demonstrated that aldehyde oxidase from rabbit liver could also catalyse the hydroxylation of a number of non-aldehydic heterocyclic compounds e.g. N-methylnicotinamide and quinine. The rabbit liver enzyme showed much greater activity than the enzyme from other mammalian species (Knox, 1946). Mahlar et al (1954) further purified the porcine enzyme and demonstrated the presence of molybdenum and iron.

Hurwitz (1955) achieved partial purification of the rabbit liver enzyme. Highly purified enzyme has been prepared from pig liver (Palmer, 1962), and rabbit liver (Rajagopalan, Fridovich and Handler, 1962).

Human liver aldehyde oxidase has been identified in post-mortem human liver (obtained within 4 hours after death) by Johns (1967). He also showed that MTX could act as a substrate for the enzyme with 7-OHMTX as the product (Johns, 1964). Using a method similar to Johns (1964), Valerino et al (1972) using DAMPA as substrate, produced a product which they suggested may be 7-OHDAMPA.

Aldehyde oxidase has been purified by homogenising the liver in ice-cold water (Hurwitz, 1955; Palmer, 1962; Johns and Loo, 1967) or in phosphate buffer (Rajagopalan et al, 1962; Johns, 1967) or in Tris buffer (Watson et al, 1978) followed by ammonium sulphate precipitation and application of the redissolved precipitate to a DEAE cellulose or Sephadex G100 column. Watson et al (1978) used a crude rabbit liver homogenate to produce 7-OHMTX by incubation with MTX standard. The mixture was extracted, the residue dissolved in the mobile-phase and injected on to an HPLC system. Blank liver homogenates were also analysed chromatographically. The chromatogram showed poor resolution and interfering peaks were present.

AIM

1. To produce mg amounts of 7-OHMTX for use as a standard.
2. To ascertain and confirm the identity of the oxidation product of DAMPA (the HPLC method was used for process control to follow the conversion of the MTX and DAMPA to their hydroxy forms).

MATERIALS AND EQUIPMENTS

Material

Sephadex G100 was obtained from Pharmacia, Uppsala, Sweden and DEAE cellulose obtained from Whatman Biochemical Limited, Springfield Hill, Kent. All other reagents were obtained from BDH Limited, Poole, Dorset, UK or as detailed on page 36.

Apparatus

An ATO mixer was obtained from MSE, England. The glass column and fraction collector were obtained from Pharmacia, Uppsala, Sweden. The freeze-dryer instrument was a 10-148 continuous MRBA type (Techmation Limited, London, UK). The HPLC instrument was as previously described.

METHODS

Crude Enzyme Preparation

A liver (freshly removed from a New Zealand rabbit) was washed first with ice-cold 40 mM Tris hydrochloride buffer, pH 7.4 containing 0.2M potassium chloride and 10 mM magnesium chloride, then intermittently homogenised using an ATO mixer with fresh ice-cold buffer (in a ratio of 1: 3.25 w/v) using 5 x 20 second to 30 second intervals. All equipment was cooled in ice during processing. The homogenised liver was centrifuged at 15K for 60 minutes at 4°C. The supernatant was removed at 4°C. This was designated the "crude enzyme preparation".

Semi-purified Enzyme Fractions

Solid ammonium sulphate was added to the crude enzyme preparation at 4°C to give a final concentration of 35% w/v. The protein was sedimented by centrifugation for 1 hour at 2000 g and, after removal of the supernatant fluid, was redissolved in water. Five ml of protein solution was applied to a 25 cm x 1 cm ID glass column containing Sephadex G100. Forty fractions of 50 drops each (15 drops = 1 ml) were eluted by 40 mM Tris hydrochloride buffer pH 7.4 containing 10 mM magnesium chloride and 200 mM potassium chloride. The fractions were monitored by UV absorption at 280 nm and the activity of the enzyme in the fractions was then determined as described below.

1. Assessment of Enzyme Activity

In order to test the activity of either enzyme preparation, 1 ml of solution was incubated at 37°C for 10 minutes with 10^{-4}M MTX solution. The protein was precipitated by heating the mixture in a boiling water bath for 5 minutes, centrifuged for 15 seconds at full speed on an Eppendorf Microfuge (Surrey, UK) and 20 ul of the supernatant applied to the HPLC column.

2. Use of HPLC for Process Control

Samples prepared as described above were analysed sequentially until the extent of conversion of substrate to product was found to be complete. Drug-free liver homogenates were incubated at 37°C for up to 6 hours and

injected on to HPLC column after deproteinisation. The HPLC method was not used for preparative work since the final product would be contaminated with ion-pair agents.

7-OHMTX Synthesis and Purification

Five hundred ml of crude enzyme preparation was incubated at 37°C with 100 mg of MTX (4×10^{-4}M) for 2 hours i.e. until complete conversion was observed. The percentage conversion of MTX to 7-OHMTX was studied at 30 minute intervals by HPLC as outlined in Figure 8, until conversion of MTX to 7-OHMTX was complete. The whole reaction solution was boiled to precipitate the protein, centrifuged at 2000 g for 15 minutes and the supernatant solution applied to 16 cm x 5.2 cm ID glass column containing DEAE cellulose.

0.6M Ammonium bicarbonate was used to elute the MTX and 7-OHMTX from the column at room temperature. The fractions were collected using a fraction collector set at 140 drops/sample and were analysed by both UV spectrophotometry (Unicam SP8000 Ultraviolet Recording Spectrophotometer, Cambridge, UK) and HPLC.

The fractions containing 7-OHMTX were pooled and freeze-dried. The identity of the samples of dried material were confirmed using mass spectrometry and nuclear magnetic resonance spectroscopy.

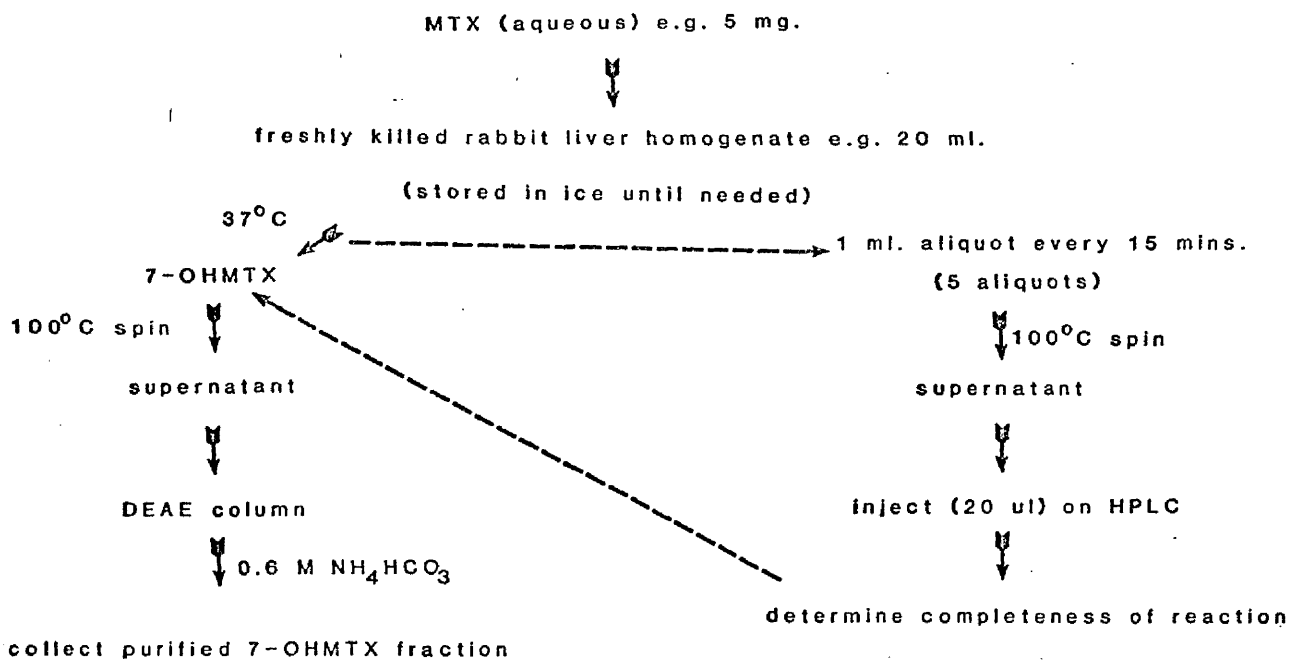


FIGURE 8 In vitro synthesis of 7-OHMTX from MTX using rabbit liver homogenate.

Synthesis and Purification of 7-OHDAMPA

Three mg DAMPA was dissolved in Tris hydrochloride buffer pH 7.4 by adding ammonium hydroxide solution to pH 8 to increase the solubility of DAMPA. Five ml of crude enzyme preparation was added to the solution and incubated at 37°C for 6 hours. A sample was taken at 30 minute intervals to study the conversion of DAMPA to its oxidation product. After 6 hours the reaction was completed, and after protein precipitation the supernatant solution applied to a 25 cm x 1 cm ID glass column packed with DEAE cellulose and eluted by 0.6M ammonium bicarbonate. Fractions were identified by UV absorption at 280 nm.

These fractions were collected, pooled and scanned on a UV spectrometer, and submitted to HPLC analysis. The pooled fractions were freeze-dried. The product of this reaction was studied by both mass spectrometry and nuclear magnetic resonance spectroscopy.

Mass Spectral and NMR Analyses

High resolution electron-impact mass spectra were obtained with an AE1 MS9 instrument. NMR spectra of MTX in deutero methanol (reference standard, TMS) and DAMPA and its metabolite in deuterium oxide containing NaOD (reference standard, tertiary butanol, δ 1.23) were recorded on a Bruker WM250 spectrometer.

RESULTS

Crude Enzyme Preparation

There was complete conversion of 10^{-4}M of MTX (2 ul = 50 ug MTX) to 7-OHMTX within 10 minutes.

Semi-purified Enzyme Fractions

The fractions collected from the Sephadex G100 column were studied for UV absorbance at 280 nm and activity of enzyme (by percentage conversion of MTX to 7-OHMTX) (Figure 9). From this Figure it can be seen that Fraction 15 contained the highest activity.

Synthesis of 7-OHMTX and 7-OHDAMPA

The HPLC chromatogram obtained from a typical reaction mixture of the initial (A), intermediate (B) and final (C) stages of the reaction are shown in Figure 10 for conversion of MTX to 7-OHMTX. Similar stages are represented in Figure 11 for conversion of DAMPA to 7-OHDAMPA. From Figures 10 and 11 it can be seen that there is complete conversion in each case. The most efficient conversions of MTX or DAMPA to their oxidised metabolites (100% in 10 minutes) were obtained when the starting concentration of MTX or DAMPA in homogenate was 50 ug/ml (1 x 10^{-4}M or 1.5 x 10^{-4}M respectively). The effect of increasing the concentration of substrate was shown to result in a lag-phase which was concentration dependent. The details for MTX as substrate are shown in Figure 12. Samples removed from drug-free liver homogenates gave no interfering peaks (Figure 13).

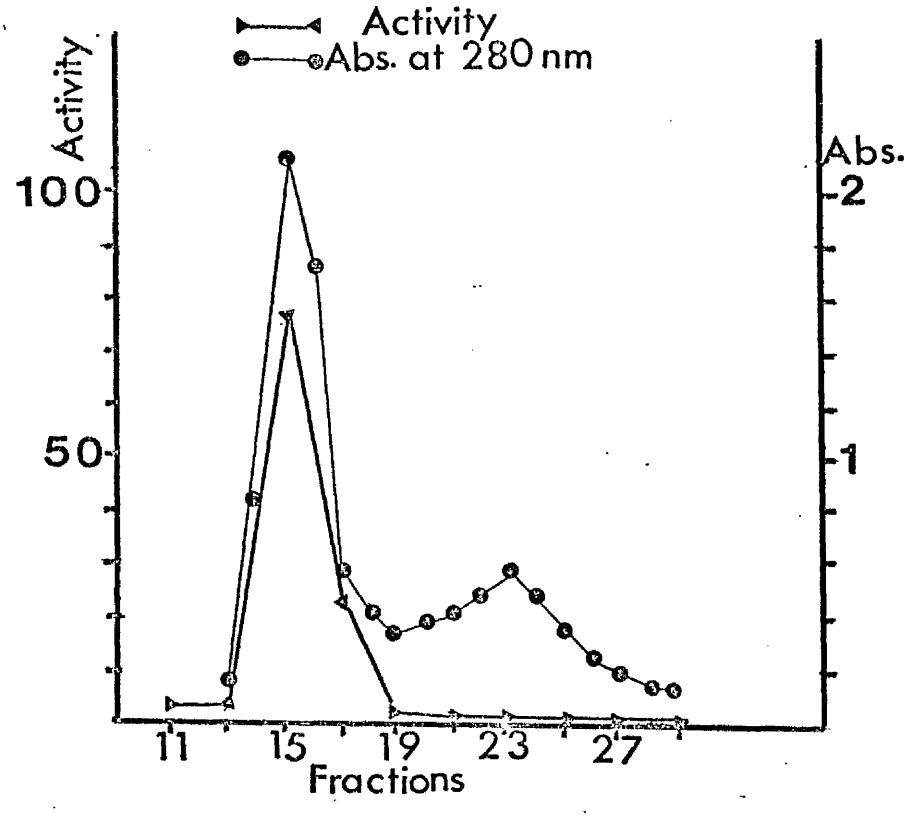


FIGURE 9 The UV absorbance at 280 nm and enzyme activity (by percentage conversion of MTX to 7-OHMTX) in fractions collected from a Sephadex G100 column.

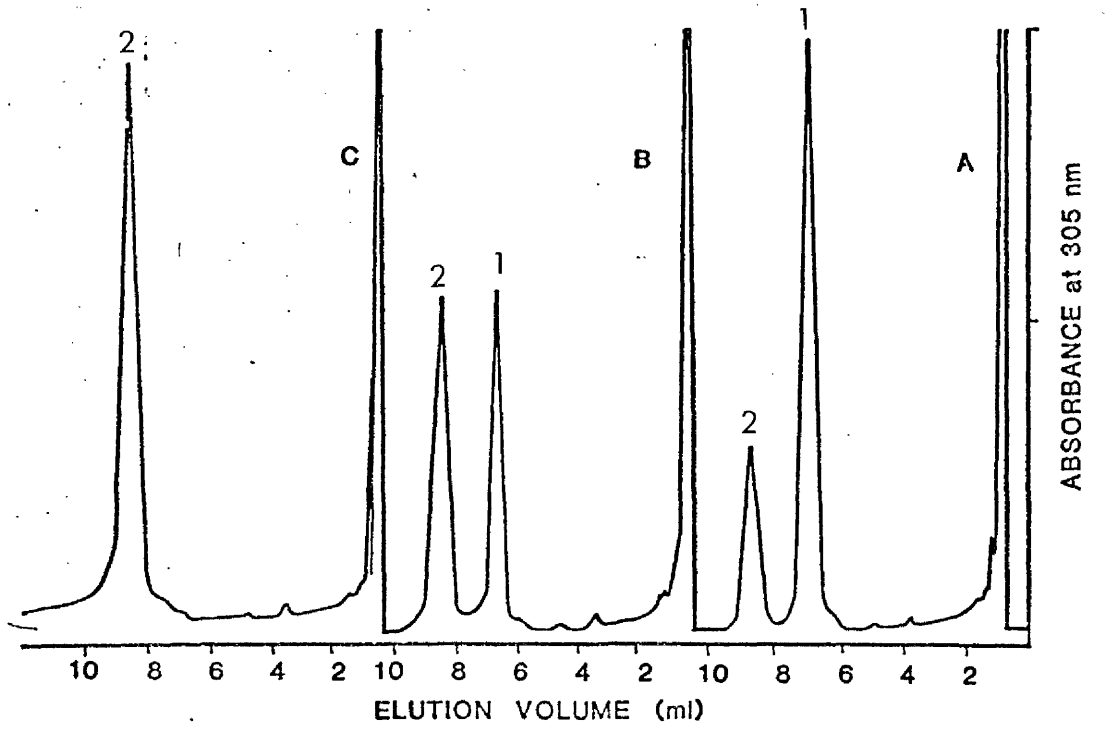


FIGURE 10 HPLC chromatogram obtained from an extract of the crude enzyme preparation + MTX at the initial (A) intermediate (B) and final (C) stages of the reaction.

Peak identities are:

1 = MTX

2 = 7-OHMTX

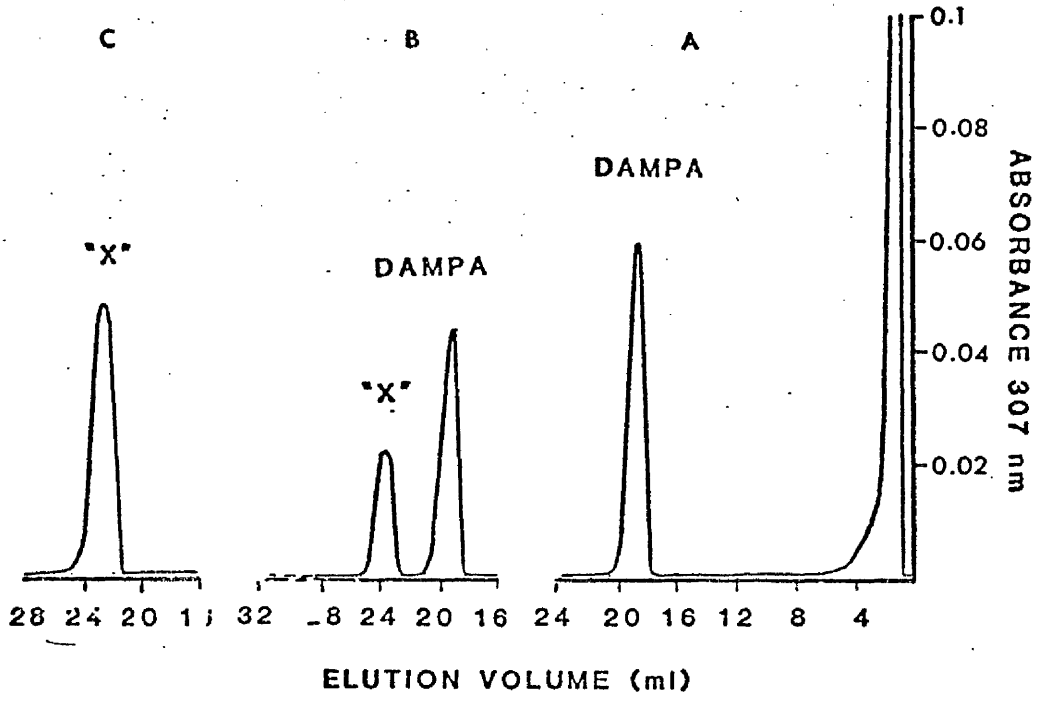


FIGURE 11 HPLC of supernatants from the reaction mixture of DAMPA incubated with crude enzyme preparation (A) initial (B) intermediate (C) final. "X" is the putative 7-OHDAMPA.

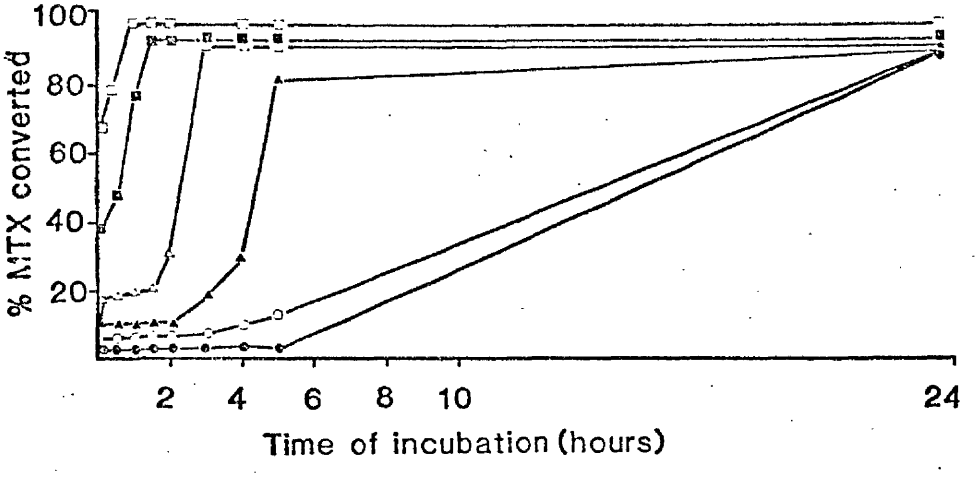


FIGURE 12

The effect of MTX concentration on the rate of conversion to 7-OHMTX by the crude enzyme preparation.

□ — □ 10^{-4}M MTX

■ — ■ 2×10^{-4}M MTX

△ — △ 5×10^{-4}M MTX

▲ — ▲ 10^{-3}M MTX

O — O 1.5×10^{-3}M MTX

● — ● 2×10^{-3}M MTX

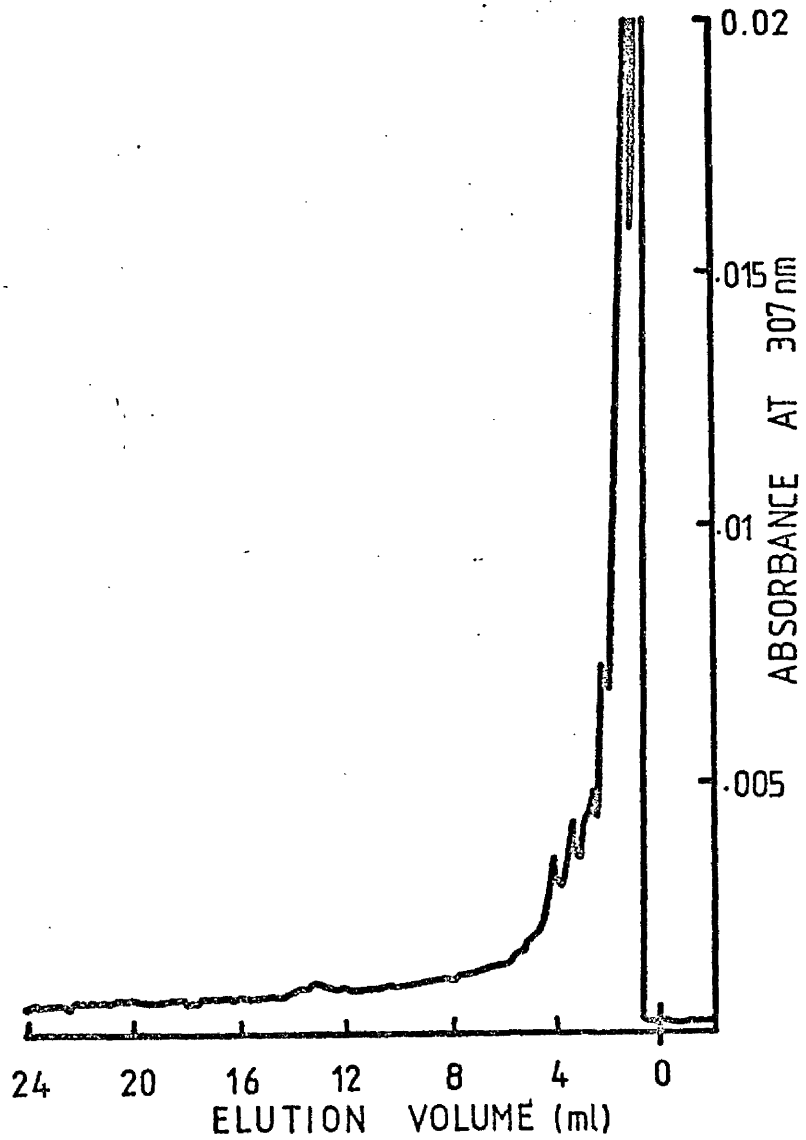


FIGURE 13 Chromatogram obtained from an extract of drug-free liver homogenate.

The use of HPLC as process control allowed a rapid optimisation of the biological synthesis of 7-OHMTX and 7-OHDAMPA.

Identification of Synthesised Products

1. 7-OHMTX

(a) UV spectrophotometry

The elution patterns monitored at 280 nm from the DEAE cellulose column are shown in Figure 14 for MTX and 7-OHMTX. The UV absorbing fractions relating to a single peak were pooled and a UV scan performed.

It can be seen that the UV scan for Fraction 100 (Figure 15) corresponded to that of the MTX standard and that the scan of Fraction 270 corresponded to that of authentic 7-OHMTX. HPLC analysis confirmed that the k' of the isolated compounds corresponded with those of the authentic compounds.

2. 7-OHDAMPA

(a) UV spectrophotometry

The elution patterns were monitored as in 1(a) above and the elution patterns for DAMPA and '7-OHDAMPA' are shown in Figure 16. Fraction 20 (Figure 17) was found to give an identical UV spectrum to authentic DAMPA. Fraction 35 was similar but significantly different with a maximium at 340 nm instead of 370 nm. This was assumed to be the oxidation product of DAMPA. HPLC analysis

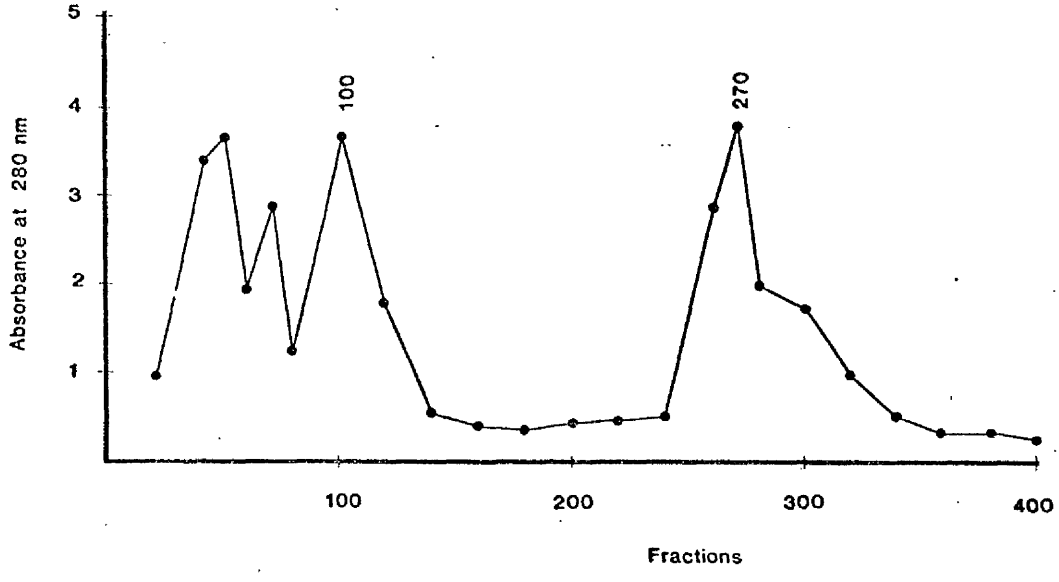


FIGURE 14 The UV absorbance of fractions from the DEAE cellulose column following addition of supernatant solution from an incubation of MTX with crude enzyme preparation.

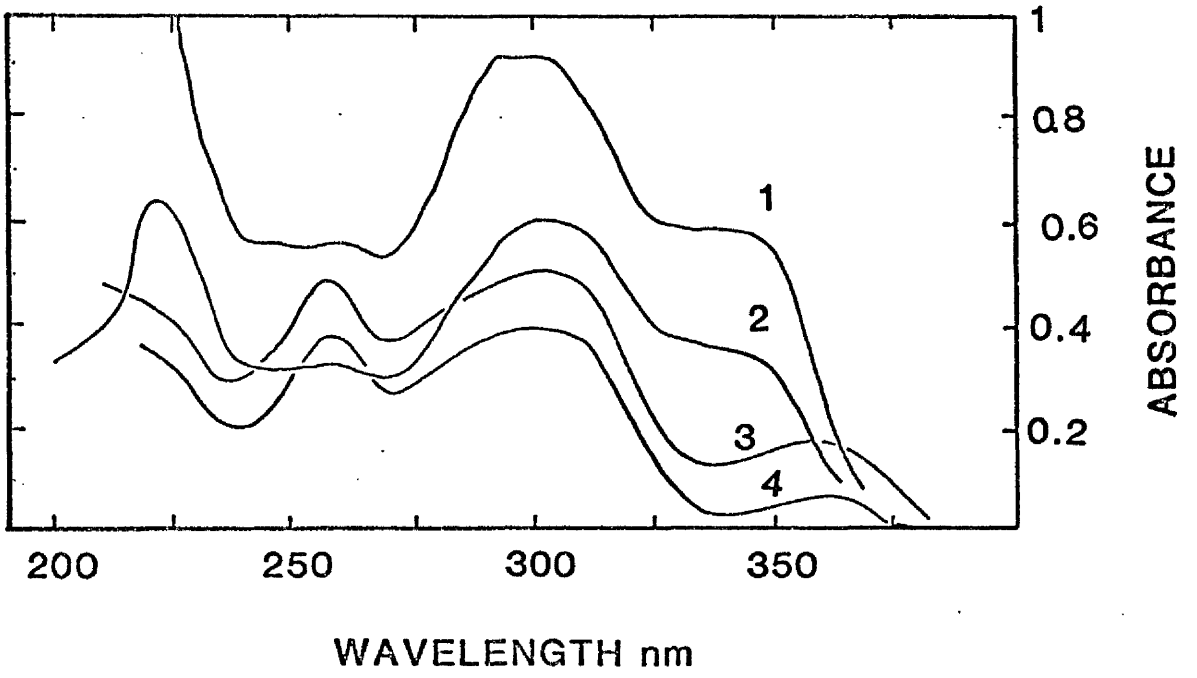


FIGURE 15 UV absorbance spectra of:

1 = Authentic 7-OHMTX

2 = Synthesised 7-OHMTX (fraction 270 from Figure 14)

3 = Authentic MTX

4 = Eluted MTX (fraction 100 from Figure 14)

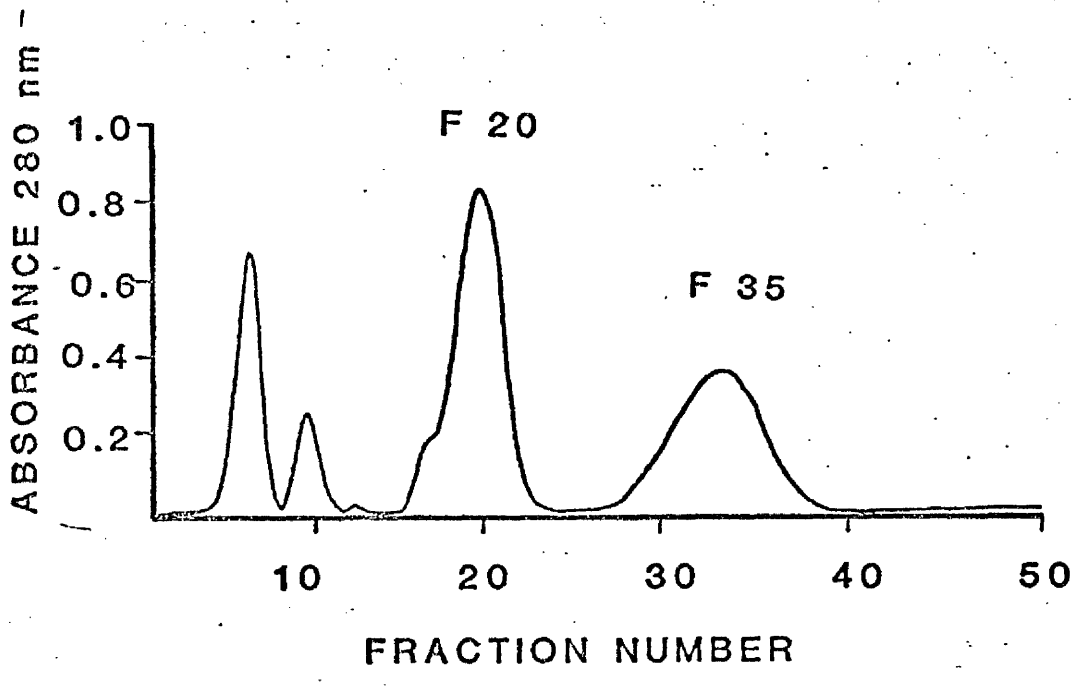


FIGURE 16 UV absorbance of fractions from DEAE chromatography of supernatant following incubation of DAMPA with the crude enzyme preparation.

F = fraction number.

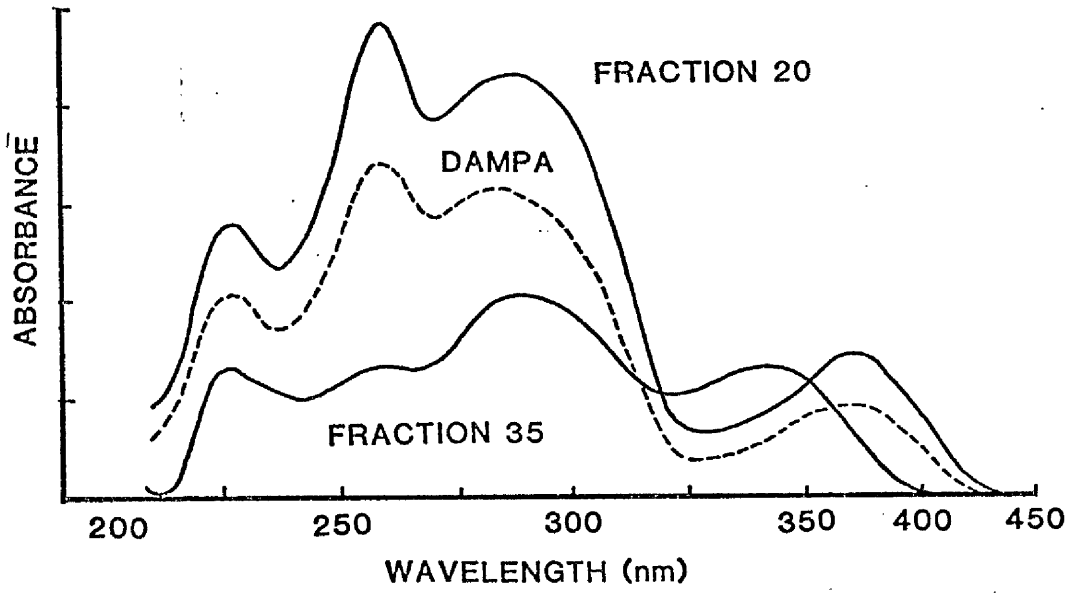


FIGURE 17 UV absorbance spectra of Fractions 20 and 35 (Figure 14) and authentic DAMPA standard in 0.6 M ammonium bicarbonate.

confirmed that fraction 20 co-eluted with DAMPA: Fraction 35 eluted with a k' = 20.6, this was designated putative 7-OHDAMPA. A chromatogram of the isolated compounds is shown in Figure 18.

The retention characteristics of putative 7-OHDAMPA relative to DAMPA compared with those of 7-OHMTX relative to MTX.

Following repeated freeze-drying, in order to remove ammonium bicarbonate, the final yield of metabolites from a complete reaction was approximately 30%. HPLC analysis of the supernatant solution, the eluant from the DEAE column and redissolved freeze-dried material indicated that 90% of losses occurred during the freeze-drying process.

(b) NMR and MS Analysis of Freeze-Dried Material

Although MTX did not give a satisfactory mass spectrum it was possible to obtain high-resolution mass spectra of DAMPA and its metabolite (Table 16) (Figure 19).

A molecular ion (M^+) observed in the mass spectrum of DAMPA; m/z 325 (14%) with fragment ions at m/z 176 and 151. The ion at m/z 176 is ascribed to the 2,4-diamino-6-pteridinyl fragment, which results from cleavage of the C^9-N^{10} bond with hydrogen radical transfer. Similar fragmentation pathways were observed for the metabolite of DAMPA, with a M^+ ion at m/z 341 and fragment ions at m/z 192 and 151. The M^+ ion had a mass 16 amu greater

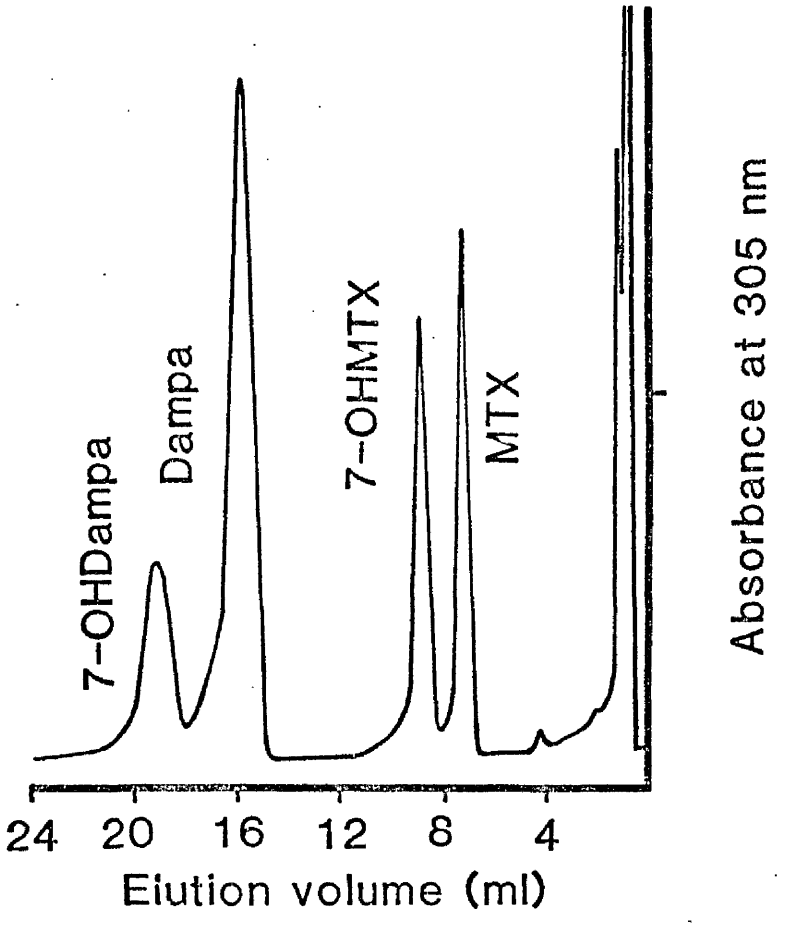


FIGURE 18 HPLC chromatogram of a mixture of standard MTX and DAMPA, 7-OHMTX and 7-OHDAMPA prepared and purified as described on pages 68

TABLE 16

High Resolution Mass Spectral Data of DAMPA and its Metabolite

SELECTED IONS

DAMPA		METABOLITE	
m/z	Formula	m/z	Formula
325.1295 (13.7%)	$C_{15}H_{15}N_7O_2$; M^+	341.1160 (1.1%)	$C_{15}H_{15}N_7O_3$; M^+
176.0809 (100%)	$C_7H_8N_6$	192.0755 (29.2%)	$C_7H_8N_6O$
151.0625 (89.9%)	$C_8H_9NO_2$	151.0572 (69%)	$C_8H_9NO_2$
150.0553 (54.8%)	$C_8H_8NO_2$	150.0559 (13.6%)	$C_8H_8NO_2$

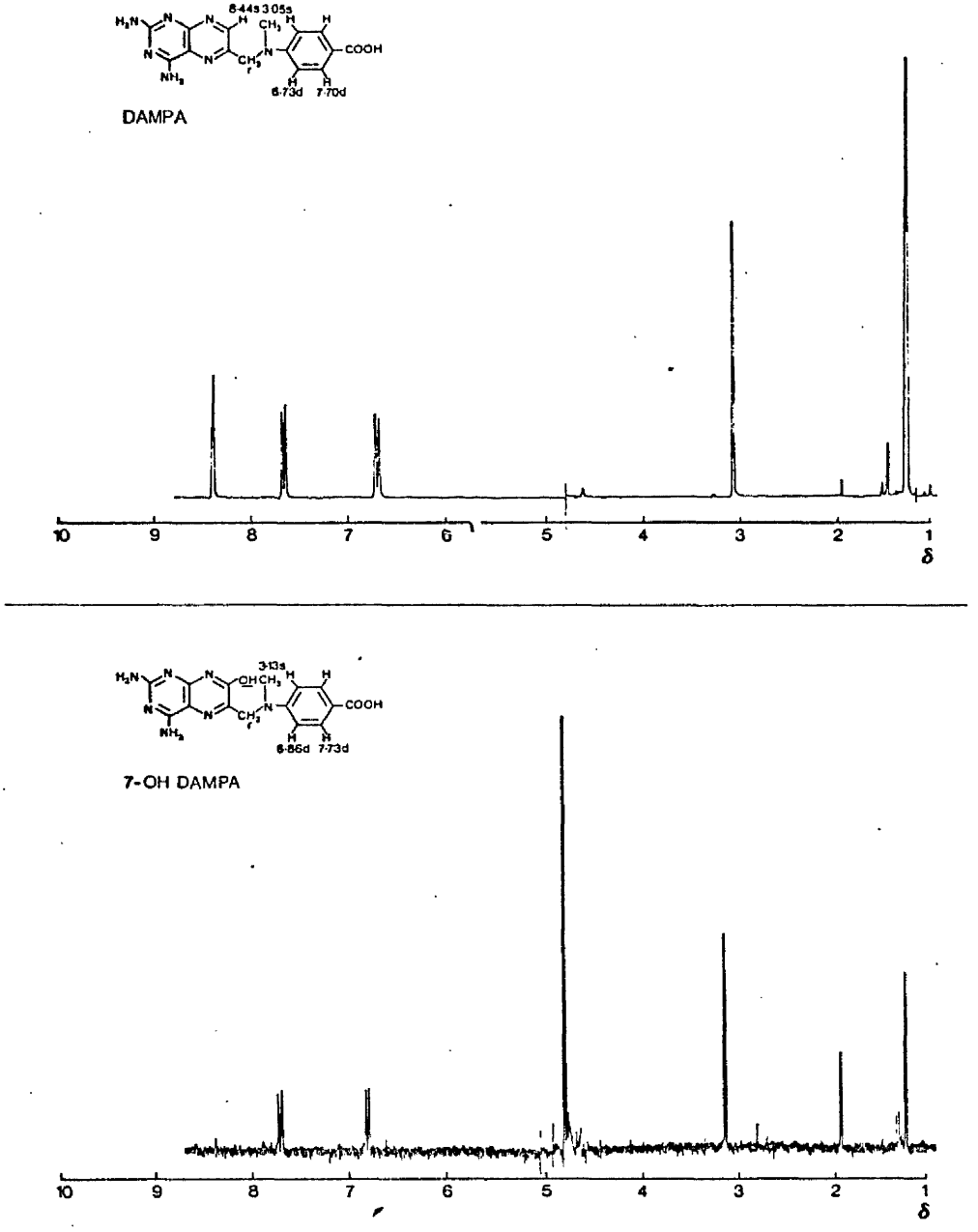


FIGURE 19 Structural assignments for NMR spectra of DAMPA and the putative 7-OHDAMPA.

S = single

d = doublet

t = triplet

r = resonance suppression

δ = chemical shift

than the M^+ of DAMPA, which is indicative of the introduction of oxygen into the molecule, possibly a hydroxyl group. That there was an ion at m/z 192 would indicate that the 2,4-diamino-6-pteridinyl moiety had been hydroxylated.

Comparison of the NMR spectra of DAMPA and its metabolite confirms that the 7-position has been hydroxylated (Figure 19), since the signal at δ 8.4 ascribed to the hydrogen at the 7-position is not present in the spectrum of the metabolite. The metabolite was thus positively identified as 7-OHDAMPA.

DISCUSSION

The initial supply of 7-OHMTX was only 3 mg (as the diammonium salt) and for further studies synthesis of more was necessary.

The use of HPLC as process control for monitoring an enzyme reaction is a relatively novel approach (Farid et al, 1983b). It has the advantage that there is minimal sample preparation, a rapid assessment of reaction progress is obtained and only a small volume of the preparation is required. Extraction methods cannot compare with this. The HPLC method also had the advantage that no interfering endogenous peaks were obtained from deproteinised incubation mixture, unlike the method of Watson et al (1978) no artifactual peaks were generated during the procedure, and it was applicable to both syntheses.

The introduction of an OH grouping into the ring results in a shift to the left of the absorption maximum at 375 nm to approximately 330 nm. A similar shift occurs with the oxidation of DAMPA.

The product of oxidation of MTX has been positively identified as 7-OHMTX, confirming the work of Johns et al (1966).

Valerino et al (1972) oxidised DAMPA and assumed that a 7-hydroxylation had taken place, this observation has not been re-examined until now. The product of this

reaction has been positively identified as 7-OHDAMPA using both mass spectrometry and nuclear magnetic resonance spectroscopy.

CHAPTER 5

CLINICAL STUDIES OF HIGH AND LOW-DOSE METHOTREXATE

INTRODUCTION

A general review of the pharmacokinetics of MTX was given in Chapter 2 for MTX in both high and low doses. Some details are extracted here for illustrative purposes.

Low-Dose

Low-dose MTX (30 mg/m^2) is well absorbed after oral administration with a peak serum level appearing after 1-2 hours. Slow absorption of small doses has been noted (Freeman-Narood, 1962). Jacobs et al (1977) reported that 7-OHMTX was not detected in samples from patients on low-dose IV therapy (< 10 mg/kg). However Chan et al (1980) reported 7-OHMTX in plasma from these patients who were given similar doses.

The cumulative recovery of MTX from urine following low-dose (< 30 mg/m^2) therapy has been reported as 80% in 24 hours (Huffman et al 1973).

The half-life of MTX has been investigated by several groups. Huffman et al (1973) reported a terminal half-life of approximately 27 hours, which conflicts with the figure of 2-10 hours reported by others (Henderson et al, 1965; Halprin et al, 1971). Lankelma et al (1980) reported that the $t_{\frac{1}{2}\beta}$ of MTX and 7-OHMTX following an IV bolus of 25 mg MTX was 5.5 hours for both, although administration of 2.2 mg 7-OHMTX to a normal volunteer resulted in a $t_{\frac{1}{2}\beta}$ of 9 hours.

High-Dose

The percentage absorption of 80 mg/m^2 MTX following oral administration was estimated as 50-70% (Henderson et al, 1965; Wan et al, 1974).

Following high-dose MTX therapy, administered intravenously, 7-OHMTX was found to be the major metabolite. DAMPA has also been detected in serum and urine of some patients treated with high-dose MTX infusion (Donehower et al, 1979; Howell et al, 1980).

About 50% of the infused high-dose MTX was recovered in urine collected between 6 and 12 hours after the end of the infusion (Stoller et al, 1975). Most users of HPLC methods (as described in Chapters 2 and 3) did not study the pharmacokinetics, an exception being Lankelma et al (1980). The majority of the pharmacokinetic parameters (reported above and in Chapter 2) were calculated following analysis using non-specific methods.

The characteristics of the HPLC method used in this study (Farid et al, 1983a) have been described earlier. The analysis of patients samples using this specific method should result in the production of more accurate pharmacokinetic parameters.

AIM

1. To determine the plasma pharmacokinetics of MTX in high and low-dose.
2. To assess the utility of urinary kinetics in high-dose therapy.

MATERIALS AND METHODS

Low-Dose Study

Fourteen patients were treated with MTX (15 mg) by both oral and IV routes. Seven male and seven female patients aged 39-81 years suffering from psoriasis were studied. Blood samples were drawn at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours after the administration of MTX. The plasma was separated and stored at -20°C until analysed by HPLC.

High-Dose Study

Six patients were treated with high-dose MTX ranging from 1.5 gm to 9 gm as an IV infusion. Details of the patients and their treatment are shown in Table 17. Samples of heparinised blood were collected at 1, 2, 3, 4, 5, 6, 8, 24 and 30 hours after commencement of the infusion. The samples were spun at 1500 g for 10 minutes. The plasma was separated and stored at 20°C until analysed by HPLC. Urine samples were collected every 2 hours for the first day and every 6 hours during the second day.

The urine volumes were measured and a 20 ml aliquot of each was stored at -20°c for subsequent analysis.

Analysis

An ion-pair chromatography method as previously described (Chapter 3) was used to quantitate the levels of MTX and its metabolites in the plasma and urine samples.

TABLE 17

The Details of Doses, Courses for Patients Treated with High-Dose MTX Therapy

Patient	Dose (gm)	Courses
R.M.	9	1
	3	3
J.S.	9	1
	3	3
D.T.	1.5	2
E.M.	1.5	2
J.M.	1.5	2
L.D.	3.25	1
	2.7	

Metabolite Identification

Urine samples in which an unknown peak was detected were pooled and stored at -20°C. The samples were loaded onto a DEAE cellulose column and eluted with ammonium bicarbonate as described in Chapter 4. The appropriate fractions were collected, scanned by UV spectrophotometry and pooled. They were then freeze-dried overnight. Further purification was then carried out by reconstituting the freeze-dried material in methanol and injecting the whole specimen onto an HPLC column in the absence of ion-pairing agents, since a metabolite free of contamination is required for NMR/MS investigation.

The fractions eluted from the HPLC column were again scanned in the UV.

Pharmacokinetic Calculations

1. Low-Dose

The pharmacokinetic parameters were calculated by using the following equations:

$$t_{\frac{1}{2}} = \frac{0.693}{k_{el}} \qquad (1)$$

$$V_d = \frac{F.D}{AUC.k_{el}} \qquad (2)$$

$$Cl = \frac{F.D}{AUC} \qquad (3)$$

Where $t_{1/2}$ = serum elimination half-life

k_{el} = serum elimination rate constant

V_d = apparent volume of distribution

D = dose

Cl = serum clearance

AUC = area under curve

F = Absorption factor

Half-lives were calculated using a semilogarithmic plot of MTX concentration versus time. The area under curve (AUC) was calculated using the trapezoidal rule.

MTX bioavailability in patients treated with 15 mg MTX either orally or intravenously in alternate weeks was calculated as the ratio of the areas under the concentration versus time curves for the oral and IV doses. The individual F values were taken into account when calculating Cl and V_d for the oral data.

2. High-Dose

The pharmacokinetic parameters were calculated as described above except that AUC was calculated from the cessation of the infusion. In addition they were derived from data obtained from analyses of urine specimens, employing the following equations:

$$\text{Urine excretion rate} = \frac{\text{Urine conc. x urine vol.}}{\text{Time}} \quad (4)$$

$$Cl_R = \frac{C_u \times U_f}{C_s} \quad (5)$$

$$f_e = \frac{\text{amount of drug excreted unchanged}}{D} \quad (6)$$

$$Cl = \frac{Cl_R}{f_e} \quad (7)$$

where Cl_R = renal clearance

C_u = urine concentration

U_f = urine flowrate

C_s = mid-point serum concentration

f_e = fraction of drug excreted unchanged

The renal clearance (Cl_R) was calculated, as was the fraction of drug excreted unchanged. The total clearance Cl was used to assess the utility of urinary kinetics by comparison with the plasma data.

The kinetics of 7-OHMTX in plasma were also calculated using equations 1-3.

RESULTS

Low-Dose Studies

1. Measurement in biological fluids

A chromatogram obtained from extracts of serum from a patient treated with low oral dose MTX at 0.25, and 2 hours after the dose (15 mg) is shown in Figures 20 and 21. 7-OHMTX was detected in some but not all patients treated with low doses (15 mg).

2. Application and bioavailability

A typical profile of MTX in the serum of a patient treated with 15 mg by the oral and IV routes is shown in Figure 22.

The bioavailabilities, calculated by comparison of AUC (oral) with AUC (IV) gave a range of 17-120% MTX absorbed in this group of 14 patients (Table 18), although the large AUC for subject A.G. in the IV study is not in keeping with the other results.

3. Pharmacokinetic parameters

The calculated pharmacokinetic parameters for MTX following low doses administered by either the oral or IV routes are illustrated in Table 19. The elimination half-lives for MTX following oral and IV routes were found to be 2.56 ± 1.3 hours and 2.48 ± 0.99 hours respectively, these were not significantly different. The peak serum levels after oral dose MTX (15 mg) appeared at 1-2 hours. There was no intra-individual

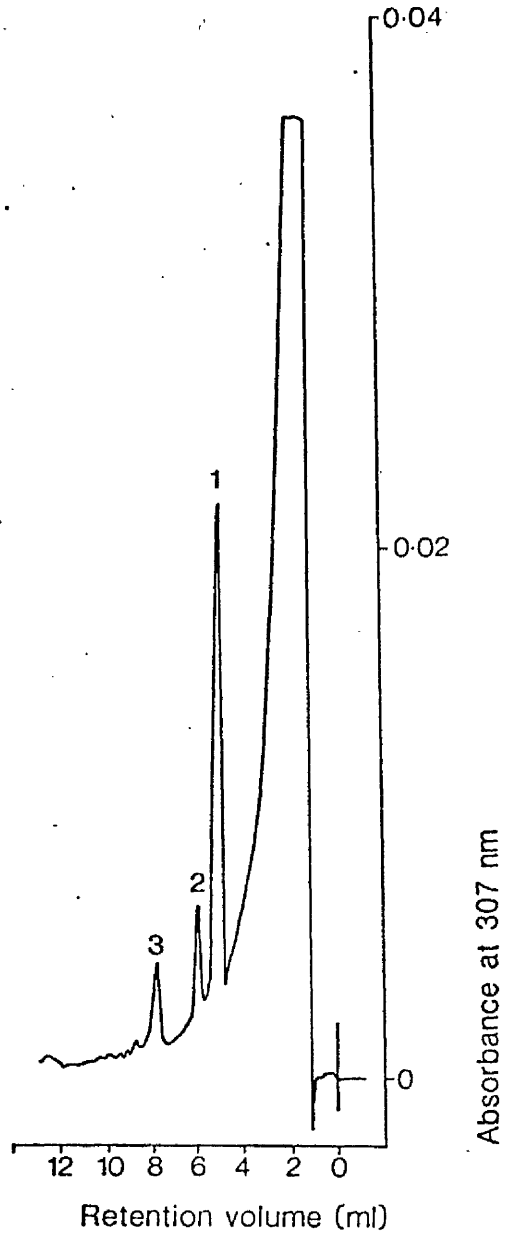


FIGURE 20 Chromatogram obtained from extracts of serum from a patient treated with low-dose (15 mg) oral MTX at 0.25 hours after the dose.

Peak identities are:

1 = L-tryptophyl L-glutamic acid (IS)

2 = MTX

3 = 7-OHMTX

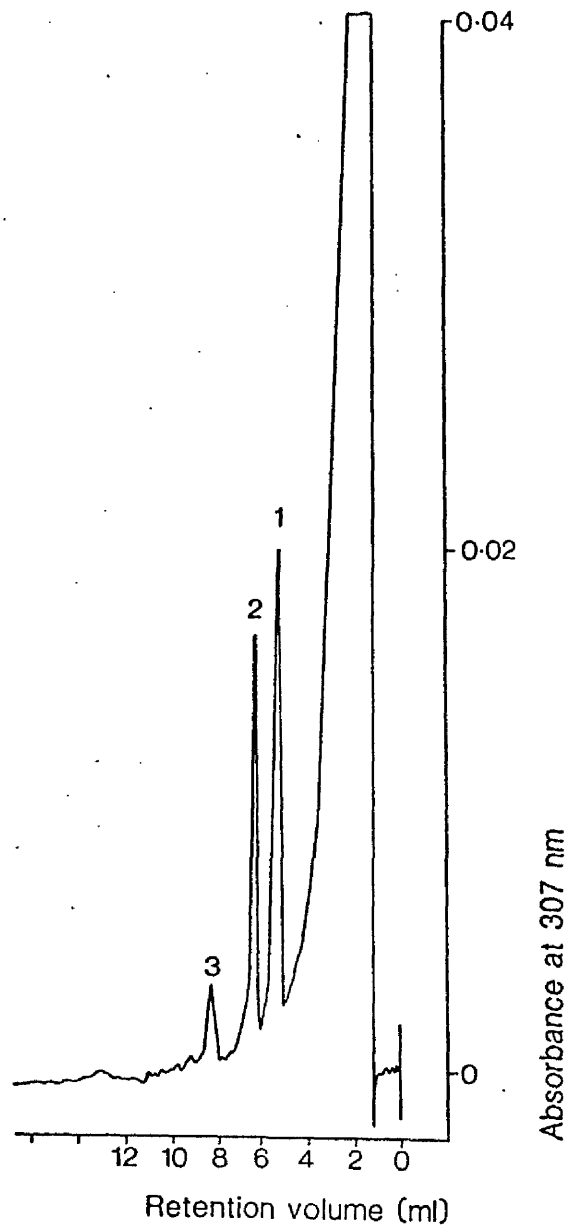


FIGURE 21 Chromatogram obtained from extracts of serum from a patient treated with low-dose (15 mg) oral MTX at 2 hours after the dose.
Peak identities as in Figure 20.

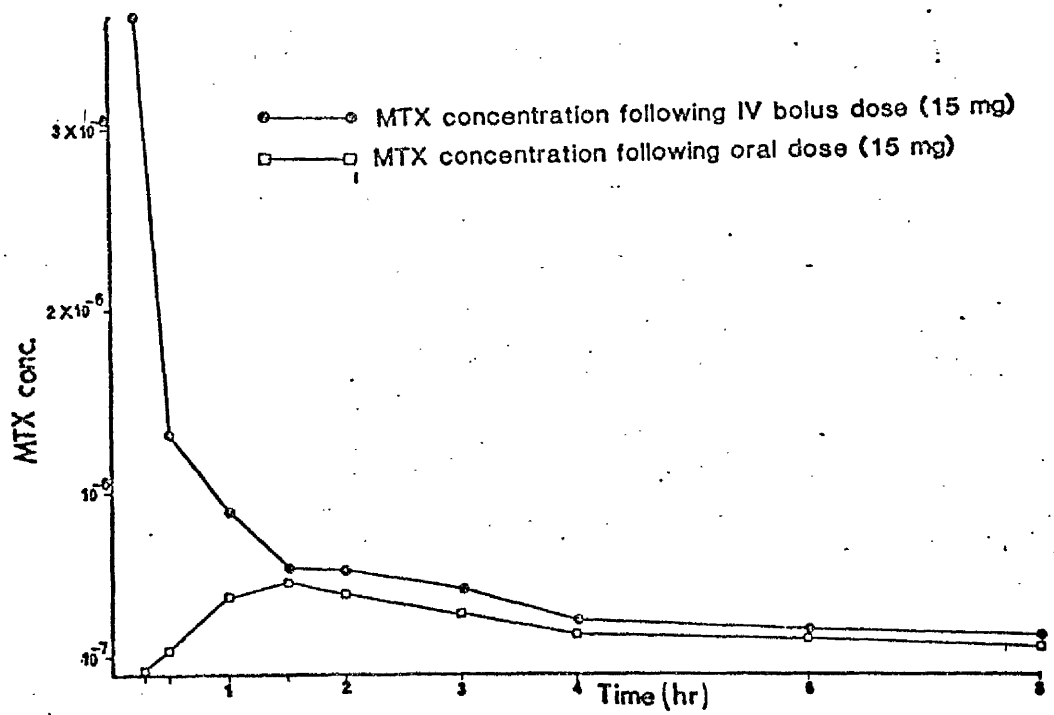


FIGURE 22 Comparison of the plasma MTX profiles following oral or IV MTX administration at low-dose in one patient.

TABLE 18

The Bioavailability for 14 Patients Treated with Low-Dose MTX (15 mg) Orally and Intravenously

Patients	Bioavailability
A.G.	17
B.G.	90
A.J.	73
A.W.	120
M.McR.	42
J.D.	17
I.C.	78
J.R.	46
E.T.	55
C.McC.	28
F.W.	89
A.A.	55
S.S.	72
P.S.	70

TABLE 19 Indvidual Pharmacokinetic Parameters for MTX following IV and Oral MTX Therapy in Psoriasis (15 mg)

SUBJECT	IV							ORAL							$F = \frac{\text{AUC Oral}}{\text{AUC IV}}$
	AUC (mg/l/hr)	$t_{\frac{1}{2}}\beta$ (hr)	K_{el} (hr^{-1})	V_d (l)	Cl (l/hr)	7-OHMTX First Detected t (hr)	nM	AUC mg/l/hr)	$t_{\frac{1}{2}}\beta$ (hr)	K_{el} (hr^{-1})	V_d (l)	Cl (l/hr)	7-OHMTX First Detected t (hr)	nM	
A.G.	9.83	2	0.347	4.4	1.5	0.5	150	1.7	2.1	0.33	4.54	1.5	0.25	150	0.17
B.G.	3.14	3	0.231	20.7	4.8	1.5	140	2.84	2.75	0.252	18.9	4.8	0.25	26	0.9
A.J.	1.67	1	0.693	13	9.0	ND	-	1.21	2.25	0.308	29.2	9.0	4.0	84	0.73
A.W.	1.49	1.7	0.407	24.7	10.o	0.5	60	1.81	1.95	0.355	28.1	10.0	0.25	100	1.2
M.McR.	5.79	3.5	0.19	13.8	2.6	-	-	2.41	3.85	0.18	14.5	2.6	0.25	78	0.42
J.D.	5.39	1.65	0.42	6.6	2.8	0.5	50	0.93	1.25	0.554	4.9	2.7	0.25	30	0.17
I.C.	1.75	3	0.231	37.1	8.6	0.5	230	1.37	2.75	0.252	33.8	8.5	0.25	100	0.78
J.R.	2.94	4.25	0.163	31.3	5.1	1.0	200	1.36	4.65	0.149	34.1	5.1	0.5	56	0.46
E.T.	1.87	4.25	0.163	49.3	8.0	1.0	80	1.04	2.25	0.308	23.8	7.9	0.5	300	0.55
C.McC.	4.71	3.1	0.223	14.3	3.2	1.5	100	1.32	5.6	0.124	25.5	3.2	0.5	130	0.28
F.W,	0.92	1.6	0.433	37.9	16.4	1.5	<10	0.82	2.2	0.315	51.6	16.3	0.25	28	0.89
A.A.	1.5	1.9	0.365	27.5	10.0	0.5	60	0.82	0.8	0.866	11.6	10.0	0.25	14	0.55
S.S.	1.15	1.9	0.365	35.8	13.1	0.5	30	0.83	1.9	0.365	35.8	13.1	0.25	30	0.72
P.S.	2.19	1.9	0.365	18.7	6.8	0.5	30	1.53	1.6	0.433	15.8	6.9	0.25	36	0.7
Mean	3.161	2.48	0.328	23.92	7.27			1.428	2.56	0.342	23.86	7.24			
Range	0.92-9.83	1.0-4.25	0.163-0.693	4.4-49.3	1.5-16.4			0.82-2.84	0.8-5.6	0.124-0.866	4.54-51.6	1.5-16.3			

variation in clearance but a great deal of inter-individual variation in both clearance and other pharmacokinetic parameters. 7-OHMTX was detected in all samples following oral administration and in 12 samples following IV administration but at a later time ($p < 0.05$, sign test).

High-Dose Study

1. Measurement in biological fluid

Representative chromatograms obtained from extracts of serum from a patient treated with high-dose MTX and a subject taking no known medication are shown in Figure 23.

The chromatograms of the equivalent urines are shown in Figures 24a and 24b respectively. The blanks are clear with no endogenous interference.

2. Presence of MTX metabolites

MTX, 7-OHMTX, DAMPA, 7-OHDAMPA were detected in the urine while only MTX, 7-OHMTX and DAMPA were detected in plasma.

DAMPA was found in 3 out of 6 patients treated with high-dose MTX in plasma and urine but was not present during every infusion.

The chromatograms obtained from serum extracts of 'early' and 'late' samples from a patient treated with a 3 gm MTX infusion are shown in Figure 25. The early sample was taken during the infusion; the MTX concentration can be seen to be higher than 7-OHMTX as

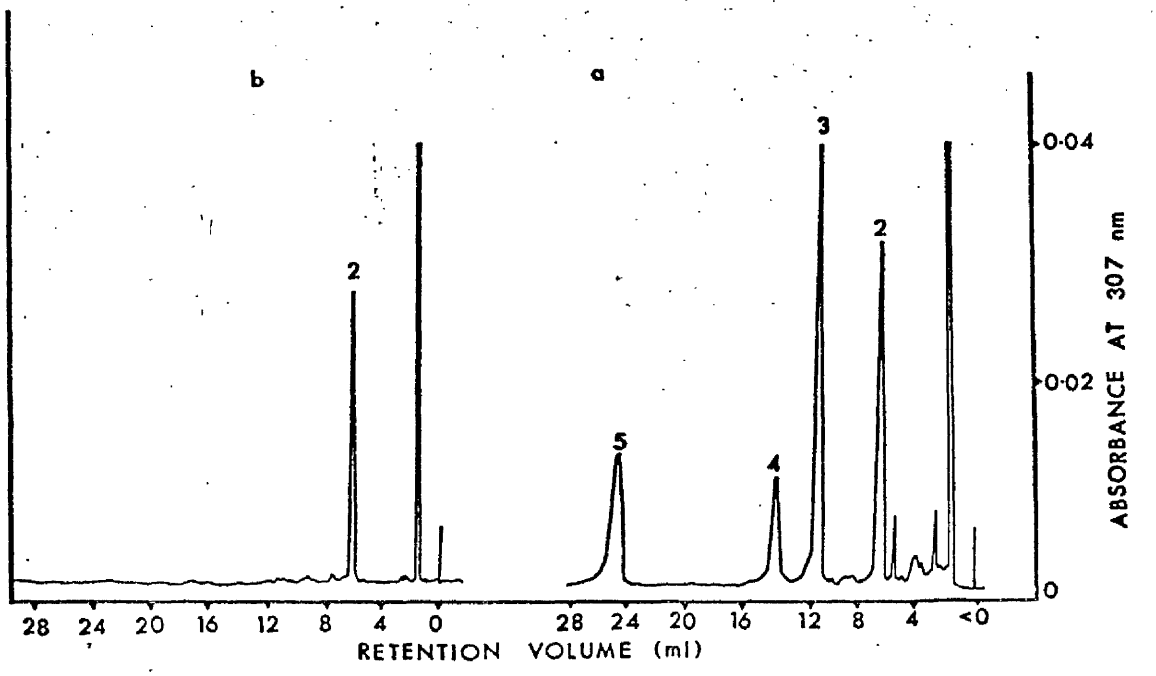


FIGURE 23 Chromatograms of serum extracts from two patients:

a) Following MTX therapy with 1 g IV bolus followed by 2 g over 24 hours.

b) Drug-free subject.

Peaks identified as follows:

2 = IS (L-tryptophyl L-glutamic acid)

3 = MTX

4 = 7-OHMTX

5 = DAMPA

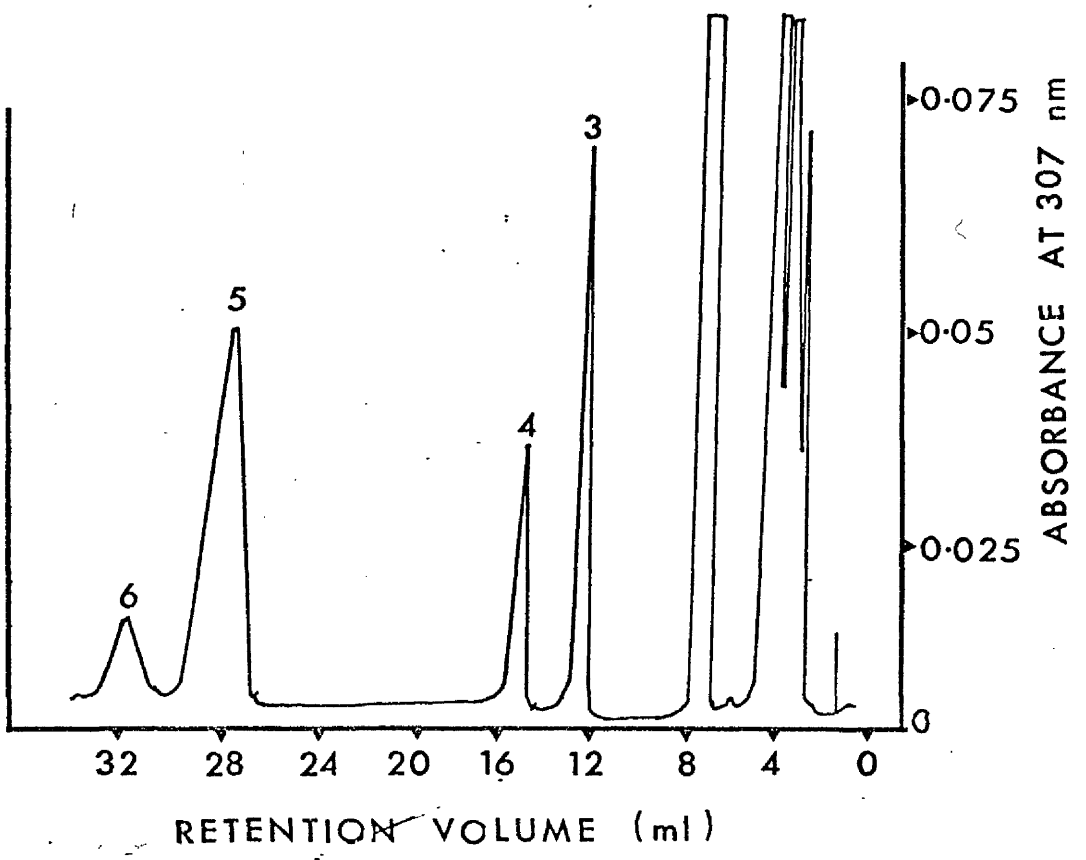


FIGURE 24a Chromatogram obtained by direct injection of 20 µl of urine from a subject receiving MTX therapy. Peaks (1-5) identified as in Figure 23.

6 = 7-OHDAMPA

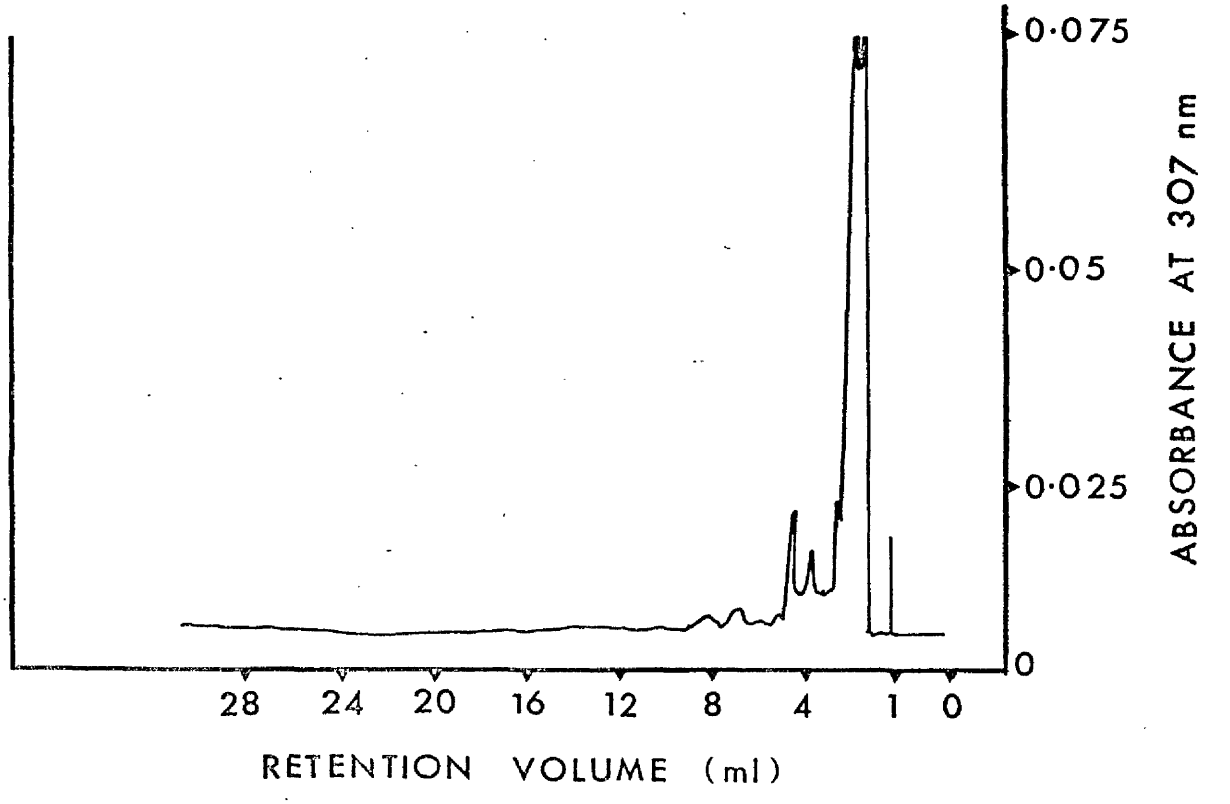


FIGURE 24b Chromatogram of a direct injection of 20 µl of urine from a drug-free subject.

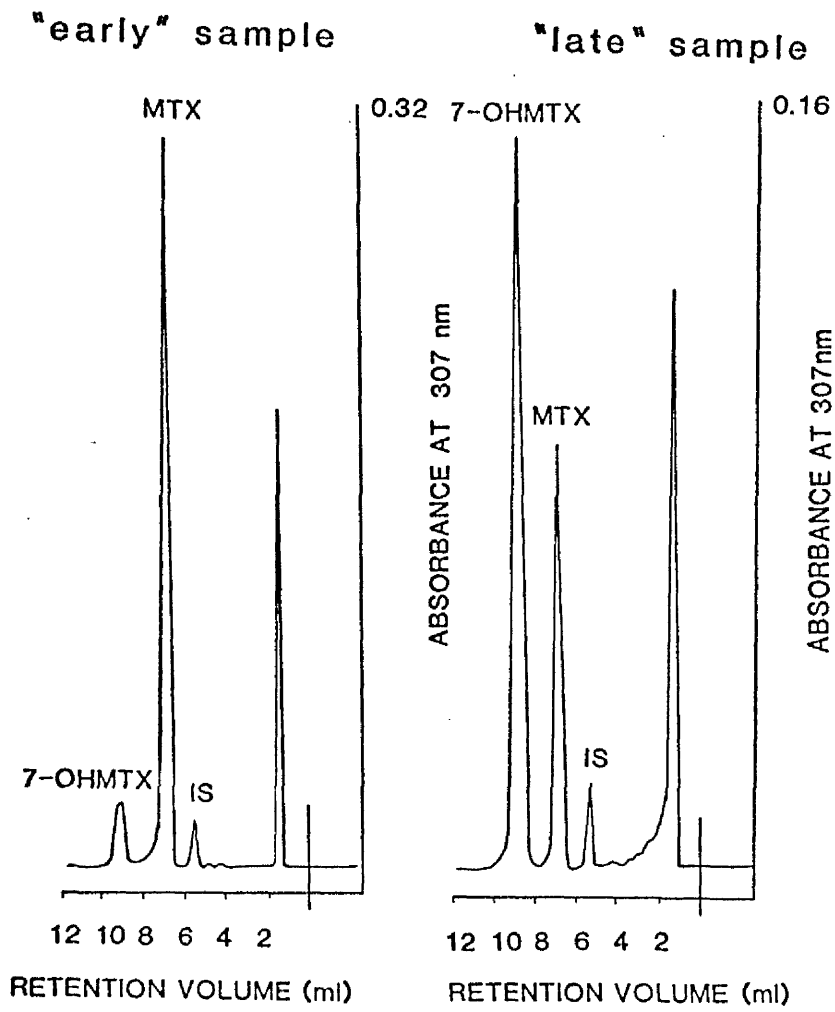


FIGURE 25 Chromatograms of serum extracts from a subject treated with 3 gm MTX by IV infusion. The "early" sample was taken during the infusion. The "late" sample was obtained 6 hours after the end of infusion.

expected. The late sample was taken at 6 hours after completion of the infusion, at which time the 7-OHMTX concentration was higher than that of MTX.

3. Application

The elimination of MTX and the rise and fall of 7-OHMTX following high-dose MTX by IV infusion is shown in Figure 26. The concentrations of 7-OHMTX were greater than those of MTX 13 hours after the dose.

The corresponding urinary time curve is shown in Figure 27. The time points represent the mid-point of each collection. There was a consistent decline in MTX concentration from 4 hours after starting this infusion. The appearance of 7-OHMTX is rapid although the concentration found fluctuated from collection to collection and may be related to intermittent alkalinisation. The pH of these urine samples was not measured.

4. The concentration of the MTX metabolites during and after sequential infusion

The concentration of MTX and its metabolites in the plasma and urine during and after 3 serial infusions with the same dose of MTX (3 gm) were studied in 2 patients. The plasma profiles following the high-dose infusion in one patient (R.M.) are shown in Figure 28. There was a significant increase ($p < 0.001$, t test) in the concentration of 7-OHMTX between the first and third infusions. DAMPA was found in the second and third courses.

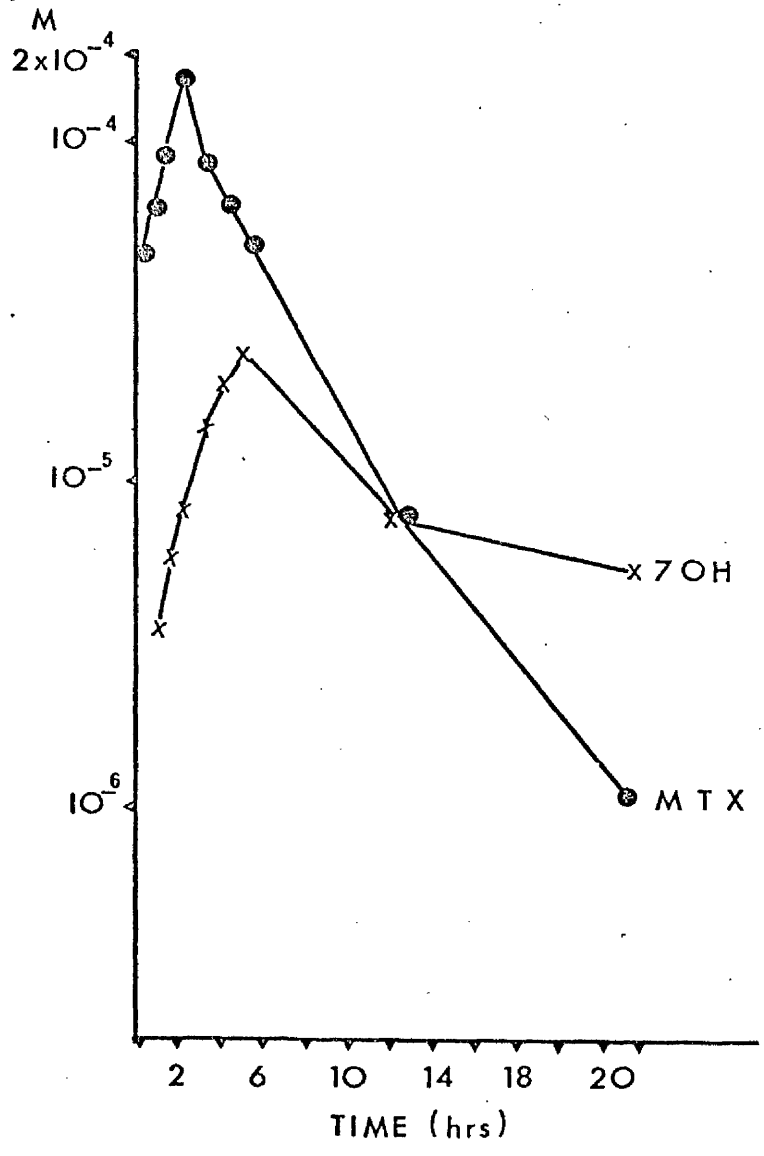


FIGURE 26 Serum concentrations of MTX and 7-OHMTX following a dose of 9 gm MTX (IV) over 6 hours.

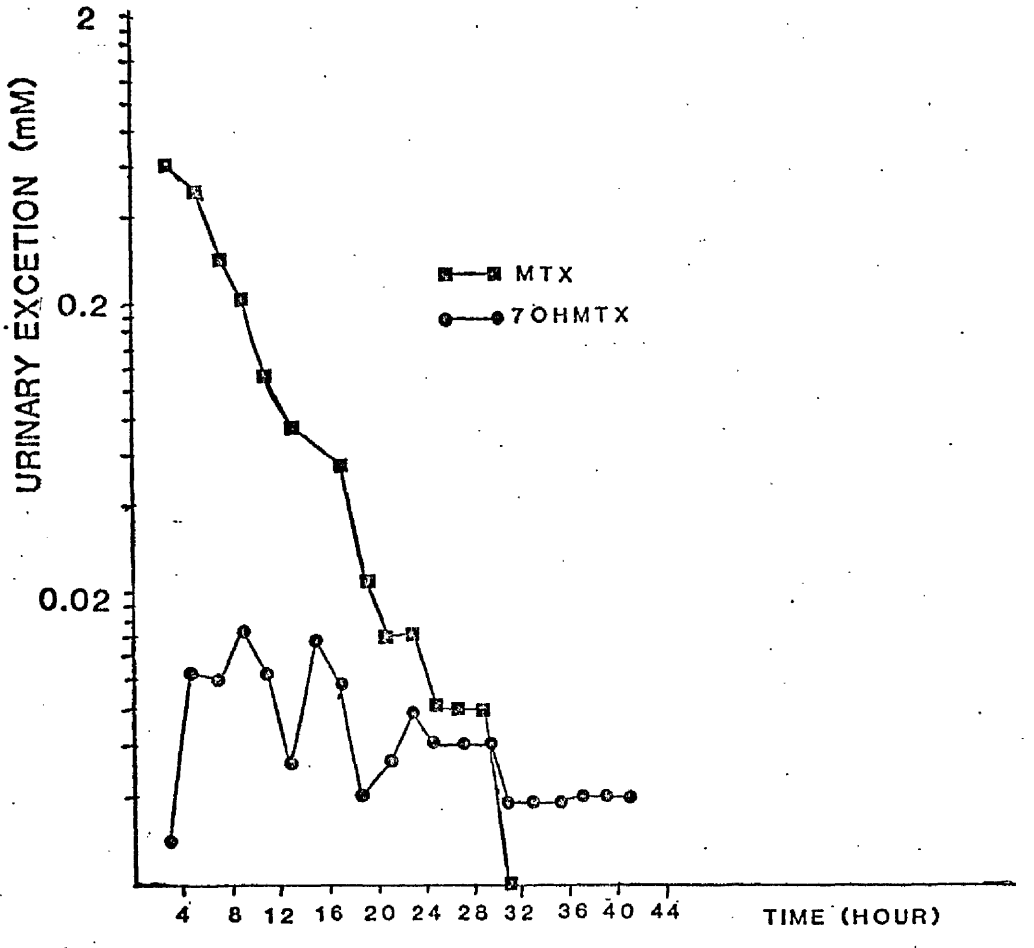


FIGURE 27 Urine concentration of MTX and 7-OHMTX following a dose of 9 gm MTX (IV) over 6 hours. The points represent the mid-point of collection.

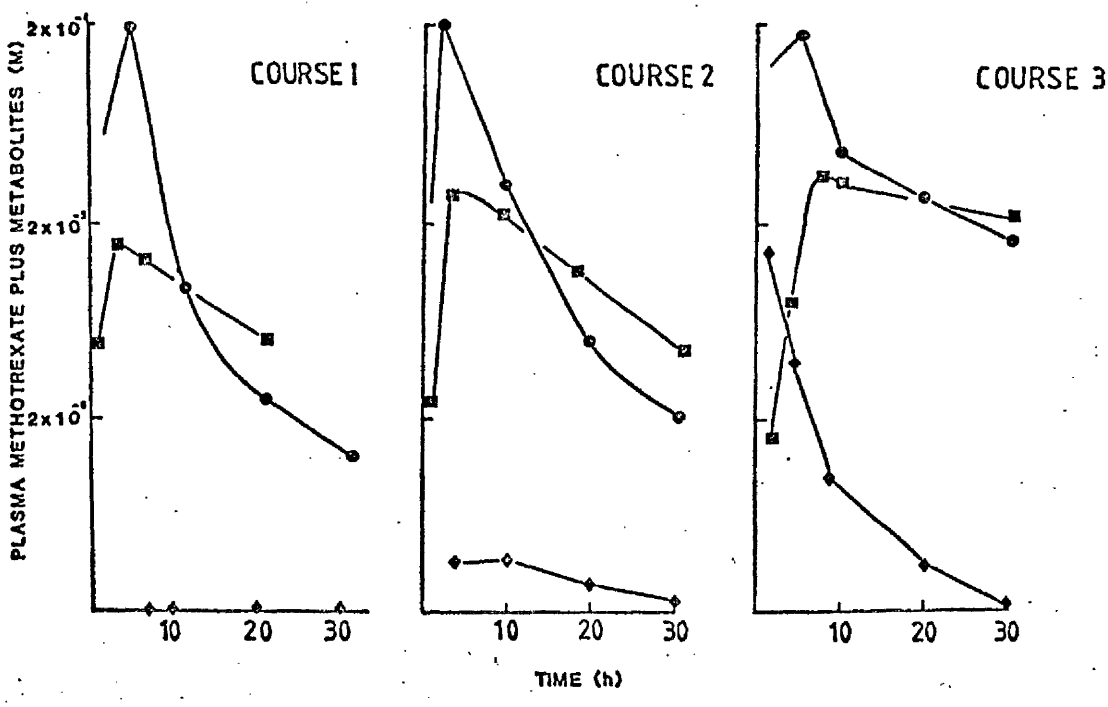


FIGURE 28 Serum concentration of MTX and its metabolites following 3 serial infusions in one patient treated with 3 gm MTX infusion over 6 hours.

● —— ● MTX

■ —— ■ 7-OHMTX

♦ —— ♦ DAMPA

The cumulative urine profiles following the high-dose infusions are shown in Figure 29. There was an apparent decrease in the 7-OHMTX excretion between the first and third infusion ($p < 0.001$, t test). DAMPA and 7-OHDAMPA were found during and following the third course.

Two serial infusions with 3.25 gm and 2.7 gm over 6 and 5.5 hours respectively were studied in a third patient (L.D.). The resultant plasma profiles are shown in Figure 30 for the first course and Figure 31 for the second course. Both 7-OHMTX and DAMPA are present on both occasions.

5. MTX excretion in urine

The mean percentage of a dose of MTX excreted in the urine in 24 hours ($\pm$1 SD) versus the MTX dose is shown in Figure 32, where it can be seen that the percentage of MTX excreted decreases as the dose increases.

6. Pharmacokinetic parameters

The pharmacokinetic parameters for MTX and 7-OHMTX following high-dose MTX infusion are presented in Table 20. These were calculated using both plasma and urine data and the clearances were comparable. Due to problems in obtaining full sets of samples, plasma data were only obtained from four courses. Two complete urine collections were obtained.

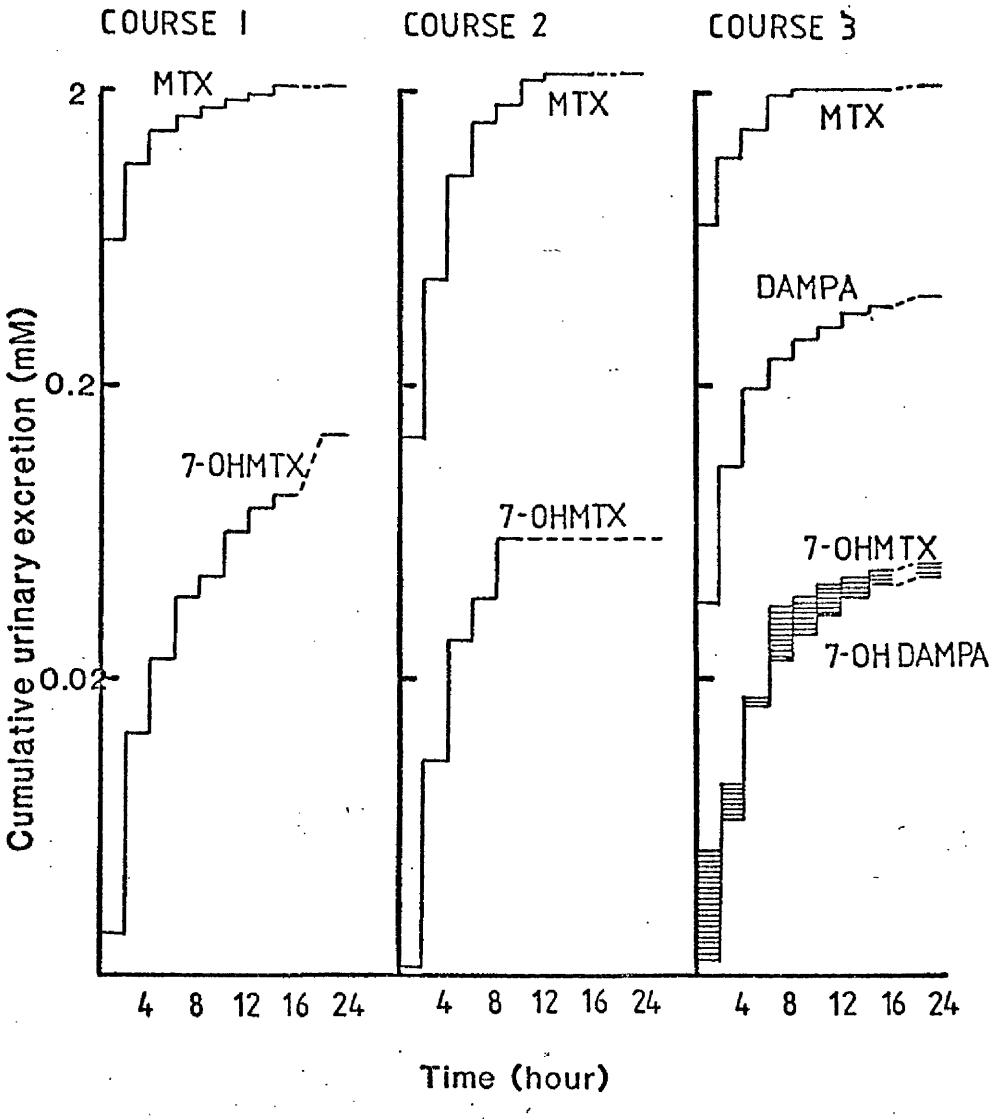


FIGURE 29 Cumulative urine excretion of MTX, 7-OHMTX, DAMPA and 7-OHDAMPA in 3 serial infusions in one patient treated with 3 gm MTX IV over 6 hours.

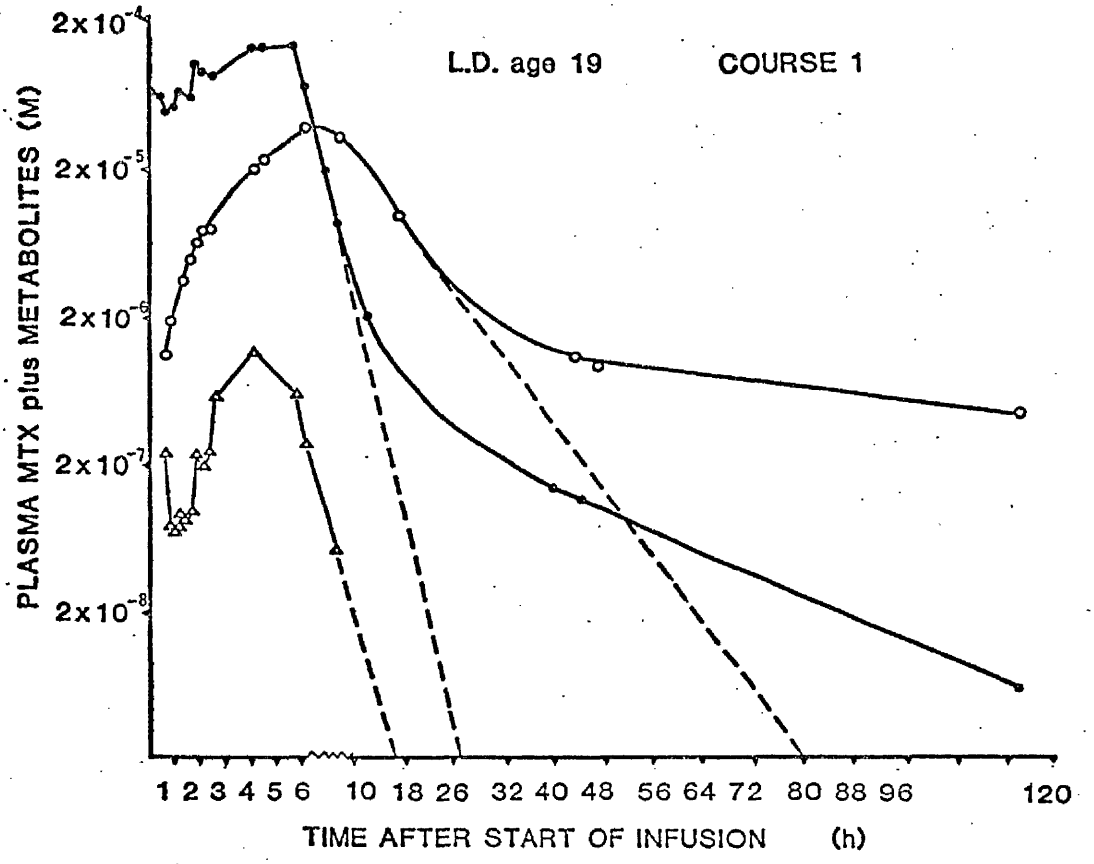


FIGURE 30 Serum concentrations of MTX and its metabolites following an IV infusion of 3.25 gm over 6 hours.

⊛——⊛ MTX

O——O 7-OHMTX

△——△ DAMPA

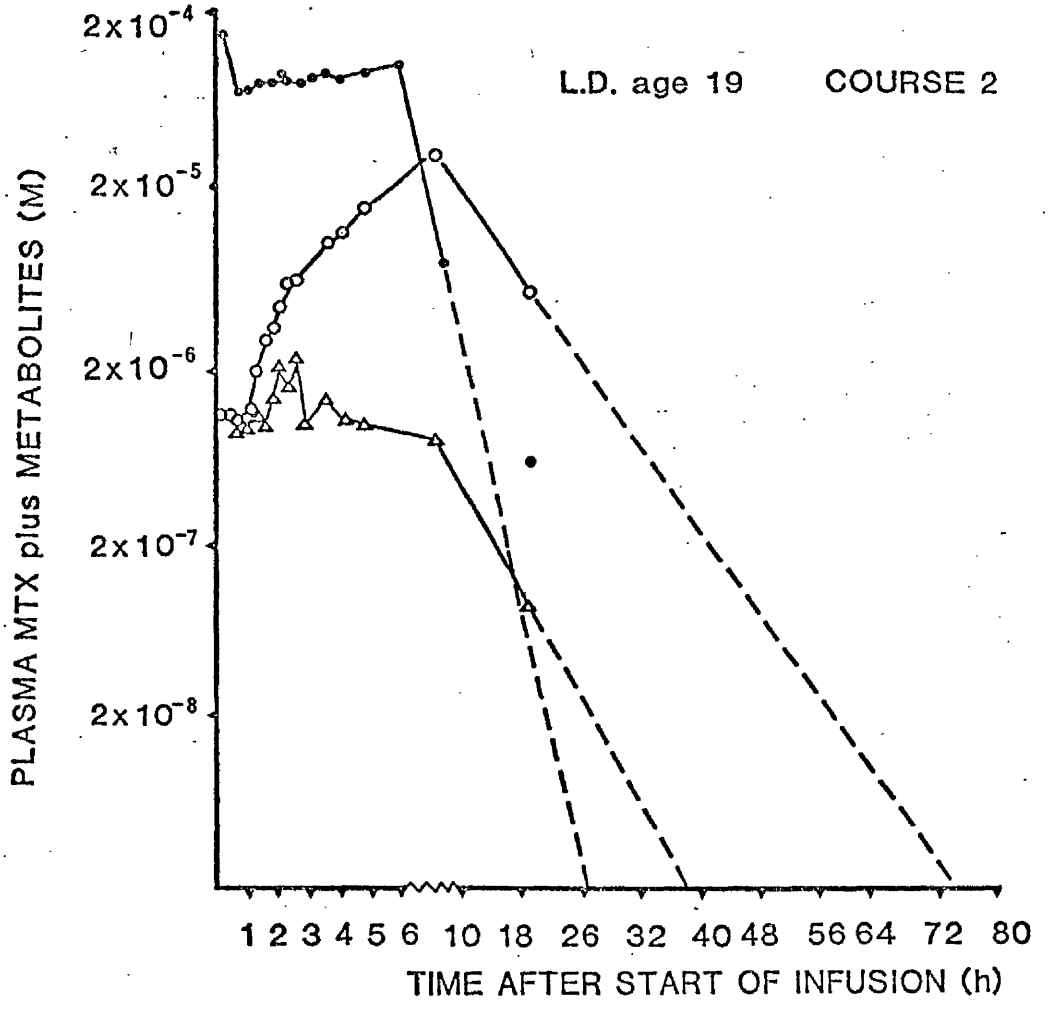


FIGURE 31 Serum concentrations of MTX and its metabolites following an IV infusion of 2.7 gm over 5.5 hours

● —— ● MTX

○ —— ○ 7-OHMTX

△ —— △ DAMPA

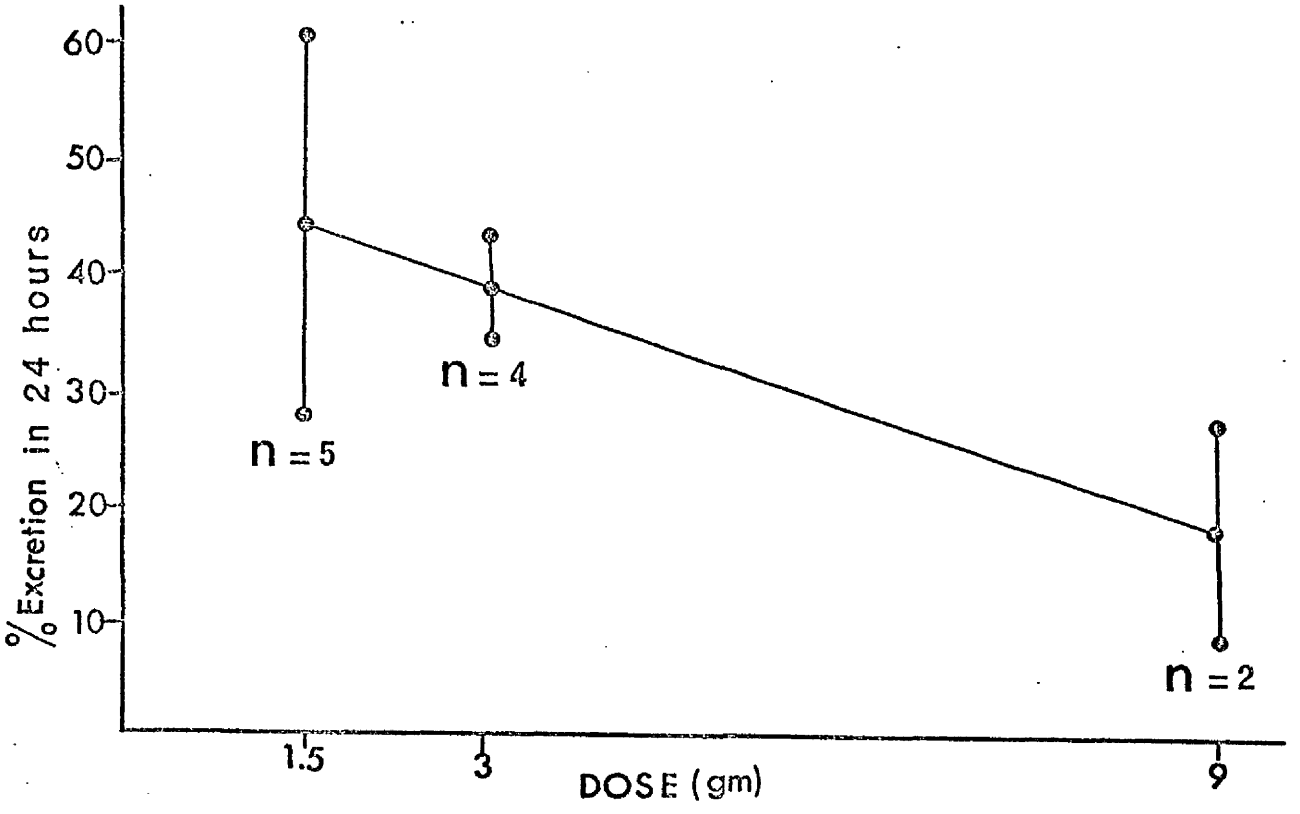


FIGURE 32 The relationship between the dose of MTX and the percentage excreted in the first 24 hours.

TABLE 20 Pharmacokinetics of High-Dose Methotrexate (MTX)

	COURSE	AUC (mg/l/hr)	$t_{\frac{1}{2}\beta}$ (hr)	V_d (l)	K_{el} (hr^{-1})	Cl Plasma Derived (l/hr)	Cl Urine Derived (l/hr)	Cl_R (l/hr)	Cl_m (l/hr)	f_e	Cr_{cl} (ml/min)
MTX											
R.M.	1	405	10.5	112	0.066	7.41	6.95	3.03	3.92	0.45	70
	2	524	8.5	72	0.08	5.73	5.44	1.86	3.58	0.34	55
	3	565	14.0	107	0.0495	5.31	-	-	-	-	50
L.D.	1	234	10.4	179	0.066	11.8	-	-	-	-	-
	Mean	432	10.85	117.6	0.065	7.56					
	±SD	148.4	1.98	44.8	0.01	2.97					
	Range	234-565	8.5-14	72-179	0.0495-0.08	5.31-11.8					
7-OHMTX											
R.M.	1	88	12	597	0.057	34.01	-	-	-	-	70
	2	204	10	212	0.0693	14.7	-	-	-	-	55
	3	347	38	480	0.018	8.6	-	-	-	-	50
L.D.	1	269	8.8	142	0.0787	11.2	-	-	-	-	-
	Mean	227	17.2	357.8	0.056	17.13					
	±SD	109	12	216.1	0.023	11.5					
	Range	88-347	8.8-38	142-597	0.018-0.787	8.6-34.01					

The AUC for 7-OHMTX increases with subsequent courses of treatment. The AUC for MTX is not clearly increased. The clearance of 7-OHMTX also declines and is related to a measured fall in creatinine clearance.

DISCUSSION

The results obtained above represent the first investigations of MTX pharmacokinetics using a highly specific method. The low-dose studies were undertaken in order to look at the effect of bioavailability in patients receiving MTX for psoriasis. These studies had not been made before since the readily available EMIT assays were not sufficiently sensitive at the levels expected ($\backsim 10^{-8}$M).

The wide range in bioavailability found (17-120%) was surprising, even accepting a proportion of error as demonstrated by the figure of 120% in one patient, and is due to a true difference in absorption of the drug, since each subject acted as his or her own control. There was no ready explanation for the AUC observed for subject A.G. in the IV study, but adoption of these values results in the calculation of a low V_d. In the absence of evidence to the contrary all values obtained were assumed to be correct.

From Table 19 it may also be seen that, even allowing for 7-fold differences in absorption, there is a considerable inter-subject variation of the clearance of MTX; clearances may vary by as much as a factor of 10. These results are confirmed by the remarkable reproducibility of clearance regardless of whether the MTX was given orally or by IV.

The problems of bioavailability therefore may be overcome by intravenous administration of MTX, however there still remains a large inter-subject variability, sufficient to cause wide differences in plasma concentration in patients receiving the same dosage.

These results confirm that for the effective achievement of reproducible plasma levels of MTX in patients receiving low-dose MTX, plasma concentrations may require to be monitored. This is particularly so since MTX is well recognised as compromising liver function as well as renal function after repeated administration.

The studies also showed that 7-OHMTX appeared in the plasma earlier and at higher concentrations following oral dosing than when MTX was given intravenously. Although the evidence is insufficient to prove it, this suggests that there may well be an element of first pass metabolism for MTX. If so, this would be the first demonstration of a first pass effect for a drug mediated by hepatic aldehyde oxidase, rather than the better understood microsomal P450 oxidation system.

7-OHMTX was also detected at 30 minutes after the initiation of high-dose intravenous MTX infusion confirming the findings of Collier et al (1982). This finding does not argue against the first pass effect proposed above. Within 30 minutes, 250 mg MTX would have been infused, assuming a hepatic extraction ratio of 0.1

and a hepatic clearance of 4 litres/hour; thus approximately 12 mg MTX would be converted to 7-OHMTX in this time; assuming distribution only in the plasma water, and assuming no renal clearance, then a concentration of 3.5 mg/l 7-OHMTX might be expected. Taking into account renal clearance and distribution this figure is considerably lower (V_d = 200 litre, Cl_R = 3 litre/hour) i.e. 12 ug/l ($\backsim 2 \times 10^{-8}$M). The concentrations at 30 minutes are of this order of magnitude.

DAMPA is detectable using this system following high-dose therapy, despite the fact that its plasma concentrations are two orders of magnitude less than MTX. The source of DAMPA has been suggested as either deglutamation of MTX by entrobacteria (Donehower et al, 1979) or as a contaminant in the infusion fluid (Chatterji et al, 1978).

DAMPA was observed in some patients during some courses of treatment, but this was not a reproducible finding. The number of data points obtained were insufficient to calculate kinetics. However, the fact that the plasma levels fell steeply in each case from the end of the infusion suggests that the source is indeed contamination of the infusion fluid, since production in vivo, especially by a route which would necessitate absorption from the gastrointestinal tract would give a curve which would have an initially rising component.

It was unfortunately not possible to obtain samples of the infusion fluids used in these courses in which DAMPA was detected. Twelve samples of infusion fluid examined prospectively contained no detectable DAMPA.

In urine samples obtained from patients with DAMPA in the plasma, a peak which co-eluted with 7-OHDAMPA, synthesised as in Chapter 4, was obtained. Attempts were made to collect and positively identify this component, however, insufficient was obtained for NMR analysis and the fragmentation pattern obtained using EI/MS was equivocal. It is possible that MS using chemical ionisation may give a final answer to this question. The metabolite did, however, show a UV spectra identical to that of authenticated 7-OHDAMPA.

The high-dose studies confirm the predominant influence of renal clearance on the removal of MTX from plasma. In Table 20, the serial results for patient R.M. indicate that a reduction in creatinine clearance is reflected in reduced MTX renal clearance. The effect on the area under the plasma MTX concentration curve is less marked. The hepatic clearance did not change significantly between serial courses of treatment.

7-OHMTX concentrations showed substantial increases in AUC in sequential courses of treatment in subject R.M. This could be related to the reduced renal clearance, but could also be due, in part, to increased conversion of MTX to 7-OHMTX due to induction. It should be noted that

induction of aldehyde oxidase activity has not been documented. There was, however, little observed fall in MTX concentrations which would be expected were induction to contribute a significant part to these findings. R.M. showed a fall in creatinine clearance of 70-50 ml/minute which could account for all of the accumulation of 7-OHMTX in this patient. 7-OHMTX is less soluble than MTX and the association noted by Link et al (1976) of reduced creatinine clearance and MTX induced renal toxicity could be the result of this.

7-OHMTX is less water soluble than MTX and is therefore also nephrotoxic. In each of the high-dose studies the concentration of 7-OHMTX exceeds that of MTX after 13 hours post infusion. This suggests that in cases where renal function is borderline it may be useful to measure this metabolite as well as MTX in an effort to determine the optimum therapy for the avoidance of renal damage.

The kinetics of MTX were calculated using both plasma and renal concentration data in order to study the possibility of using the non-invasive method. The similarity of the results obtained using the two methods (Table 20) means that urinary studies may be used for the calculation of total clearance and other parameters.

In particular the figures obtained for R.M. showed that the renal clearance decreased during the course of three infusions, whereas non-renal (mainly hepatic) clearance was unchanged.

In the low-dose study it was noted that there was wide inter-individual variation in MTX handling. Mojalano et al (1979) have suggested that a low-dose study should therefore be used in order to assess the dose required for high-dose therapy. However, a comparison of the low and high-dose kinetics shows that there was a significant increase in $t_{\frac{1}{2}\beta}$ in the high-dose patients over those receiving low doses, of the order of 5 to 10-fold.

The dose-related excretion of MTX, with a lower proportion of the dose excreted at high doses holds, whether or not 7-OHMTX is added. So far as is known this fact has not been highlighted before.

Following high doses, higher volumes of distribution were observed. Part of this in patient L.D. can be related to the presence of a third space in the form of a pleural effusion, and was probably also the case with R.M.

At low doses there is saturation of the primary intracellular binding sites (thought to be in part DHFR) which is overcome by less selective binding at higher doses (Dedrick, Zaharko and Lutz, 1973). These facts could account for the differences in calculated parameters.

In view of the variability in the pharmacokinetics in the studies of both high and low-dose MTX, the significantly lower solubilities of the MTX metabolites and the implications of this for patients, it may be necessary not only to follow the concentration of MTX during high-dose therapy but also to determine 7-OHMTX.

MTX plasma concentrations during low-dose therapy may also be of assistance in optimising oral dosage.

CHAPTER 6

IN VITRO STUDIES OF THE INTERACTIONS BETWEEN METHOTREXATE AND BACTERIAL AND HUMAN CULTURED CELLS

INTRODUCTION

Bacteria

A loss in biological activity of MTX after its contact with several kinds of bacteria was first reported by Webb (1955). Levy and Goldman (1967) reported the conversion of MTX to its deglutamated metabolite, DAMPA, using pseudomonads isolated from soil. This metabolite has also been detected in man, and it has been suggested that it might be produced in the intestine by the action of bacterial carboxypeptidase (Donehower et al, 1979).

Zaharko, Bruckner and Oliverio (1969) reported that in the mouse and rat significant amounts of previously undetected MTX metabolite were present in urine and faeces after intraperitoneal administration of the tritium-labelled MTX. The production of this metabolite was significantly less in germ-free mice and also in mice which had been pretreated with antibiotics, it was suggested therefore that intestinal bacteria were responsible for the conversion. Valerino et al (1972) identified the metabolite as DAMPA. There are no reports of studies on intracellular metabolites in bacteria.

Human Culture Cell Lines

The metabolites of MTX inside a variety of cultured cells have been studied. Rosenblatt et al (1978) reported the presence of $MTXG_1$ and $MTXG_2$ in cultured human fibroblasts. These metabolites were identified and eluted from Sephadex G15.

Whitehead (1977) synthesised the MTX polyglutamates in L1210 murine leukaemic cells and used Sephadex G15 for eluting those metabolites. Gewirtz et al (1979) used DEAE cellulose to identify the MTX polyglutamates in rat hepatocytes. Most of the authors used labelled MTX for their studies. In this work, the effect on MTX of several species of enterobacteria and a group of cultured cell lines was studied by analysing both the supernatant medium and the cytoplasm using the HPLC method previously described (Chapter 3).

MATERIALS AND METHOD

Bacteria

The strains were grown in Mueller-Hinton broth. These bacteria strains were: Citrobacter Freundii, Klebsiella Pneumoniae, Pseudomonas Aeruginosa, Acinetobacter Calcoacetius (Var. Anitratrium), Escherichia Coli (E. Coli), Klebsiella spp and Pseudomonas spp.

The bacterial cultures were incubated in broth with different concentrations of MTX (10^{-6}M, 10^{-5}M and 10^{-4}M). Blank samples were obtained from cultures containing no MTX. After incubation for 24 hours the tubes were centrifuged at 1500 g to sediment the bacteria and the supernatant broth stored at -20°C until analysed. The bacterial pellets were washed twice with normal saline and stored at -20°C.

1. Supernatant

One ml of supernatant broth was heated in a boiling water bath for 5 minutes. Following centrifugation, 20 ul of the clear supernatant was injected onto the HPLC column using the HPLC method previously described (Chapter 3).

2. Bacterial Pellets

The residue was resuspended in 10 ml distilled water and sonicated using an ultrasonic probe for 10 minutes. The solution was cooled in ice during sonication. The pH of the solution was adjusted to pH8 using potassium carbonate solution and then applied slowly to a column of Dowex 1-x2 (2 cm in height). One hundred ug of L-tryptophyl L-glutamic acid (internal standard) was mixed with the solution before application to the resin.

Four ml of methanol was used to wash the resin and the eluant was discarded. MTX and its metabolites were eluted using 2 ml of 5% acetic acid in methanol and 4 ml of 25% acetic acid in methanol. The acidic fractions were collected and were evaporated to dryness using the rotary evaporation vacuum pump. The residue was dissolved in 100 ul mobile-phase and 20 ul was injected onto the HPLC column.

Cells

A variety of human melanoma and choriocarcinoma cells (Table 21) were grown in culture medium containing different concentrations of MTX or 7-OHMTX by Gaukrodger et al (1983). The details of the procedure were as follows.

The cells were grown in RPMI 1640 medium supplemented with 10% v/v newborn calf serum (Flow Laboratories, Irvine, Scotland). Different batches of serum containing folate at concentrations ranging between 25-53 nM were used. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2/95% air. The density of the cells was 5×10^4 cells per 25 cm^2 tissue culture flank. The tissue cultures were inoculated in duplicate with MTX or 7-OHMTX at concentrations of 1×10^{-6}M.

Samples from cultures were taken after 36 hours and 11 days for HPLC analysis The incubation samples were centrifuged and the cells were separated from the supernatant. The cells were washed twice in serum-free medium and then suspended in 1 ml distilled water and ruptured using an ultrasonic probe for 20 seconds.

TABLE 21

Human Melanoma and Choriocarcinoma Cells Studied

Human Melanoma	B8
	B1O
	MEL 57
	MYJ 15
	ADLER
	Kotler
Murine Melanoma	PG 19
Human Choriocarcinoma	BeWO
	JAR

1. Supernatant from cell cultures

The supernatant was boiled in a heated water bath for 10 minutes to precipitate the protein. Following centrifugation, 20 ul of clear supernatant was injected onto HPLC using the method previously described in Chapter 3.

2. Cell pellets

Following rupture of the pellets, the solution was extracted using the anion-exchange resin with L-tryptophyl L-glutamic acid as internal standard. The residue was dissolved in 50 ul mobile-phase and 20 ul was injected onto the HPLC column using the same method as previously described in Chapter 3.

RESULTS

Bacteria

1. Supernatant broth

There were no significant changes in MTX observed in any of the broth samples. DAMPA was the only metabolite that was expected (Donehower et al, 1979), but it seems that none of the bacteria that were used in this protocol were capable of forming DAMPA in vitro.

2. Bacterial pellets

Chromatograms of the extract obtained from one strain of E. Coli after incubation with different concentrations of MTX (10^{-5}M and 10^{-4}M) is shown in Figure 33a, b. It can be seen a peak with an identical elution to MTX triglutamate ($MTXG_2$) was found in each case. The chromatograms obtained from other bacterial species showed no formation of such a metabolite. An example is shown in Figure 34.

Cells

1. Supernatant from cell cultures

The supernatant culture media from cells incubated alone, or treated with either MTX or 7-OHMTX for 7 days or 11 days were analysed by HPLC. Incubation was continued to 11 days in order to investigate a possible relationship between cell lysis and MTX metabolism. No correlation was found.

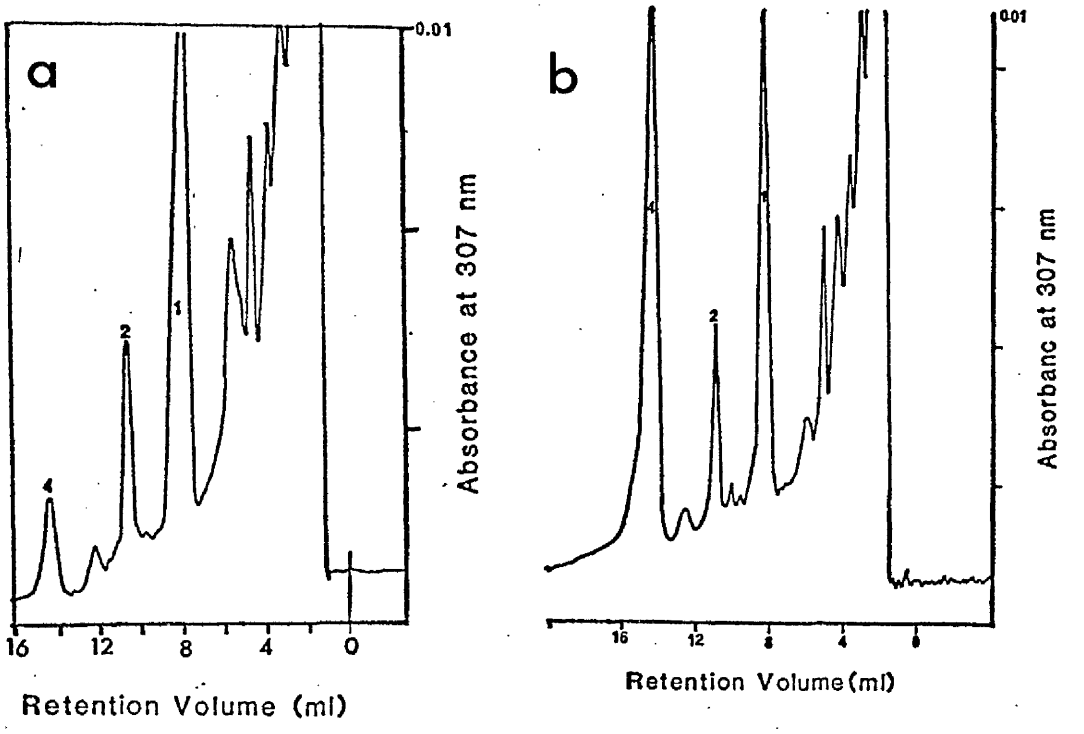


FIGURE 33 Chromatogram obtained from an extract of culture medium when E. Coli was incubated with (a) 10^{-5}M MTX and (b) 10^{-4}M MTX.

Peak identities are:

1 = Internal standard

2 = MTX triglutamate (G_2)

4 = MTX

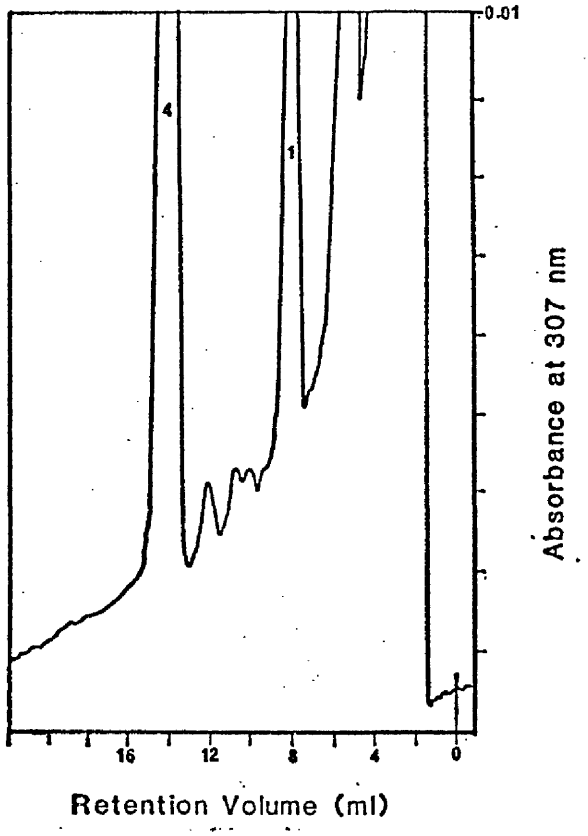


FIGURE 34 A chromatogram obtained from the culture medium following incubation of pseudomonas aeuroginosa with MTX showing no formation of $MTXG_2$.

Representative chromatograms obtained at 11 days from PG19 cells which metabolise both MTX and 7-OHMTX, and from B10 cells which metabolise neither compound are shown in Figures 35a, b. In each experiment chromatograms from 'control' cultures, incubated for the same time intervals in the absence of MTX and 7-OHMTX showed only the peak marked 'M'. In each case the baseline was flat in the area of the metabolite peaks, at a detector sensitivity of 0.08 AUFS. Supplementation of culture extracts with MTX or 7-OHMTX immediately prior to HPLC gave peaks eluting in the region of the profile assigned to the pure compound.

These studies showed that some of the melanomas appeared to metabolise added MTX. The findings are summarised in Table 22.

In the PG19 cultures the metabolites appeared either as a shoulder on the main MTX peak or as additional peaks in the optically active material eluting early from the column (Figure 35a). These peaks do not correspond to any of the known metabolites. They did not co-chromatograph with polyglutamyl derivatives of MTX. The patterns were reproducible within cell lines. Studies with 7-OHMTX show that this was more stable in the cultures and as can be seen from Figure 35a did not yield the same metabolite profiles as MTX.

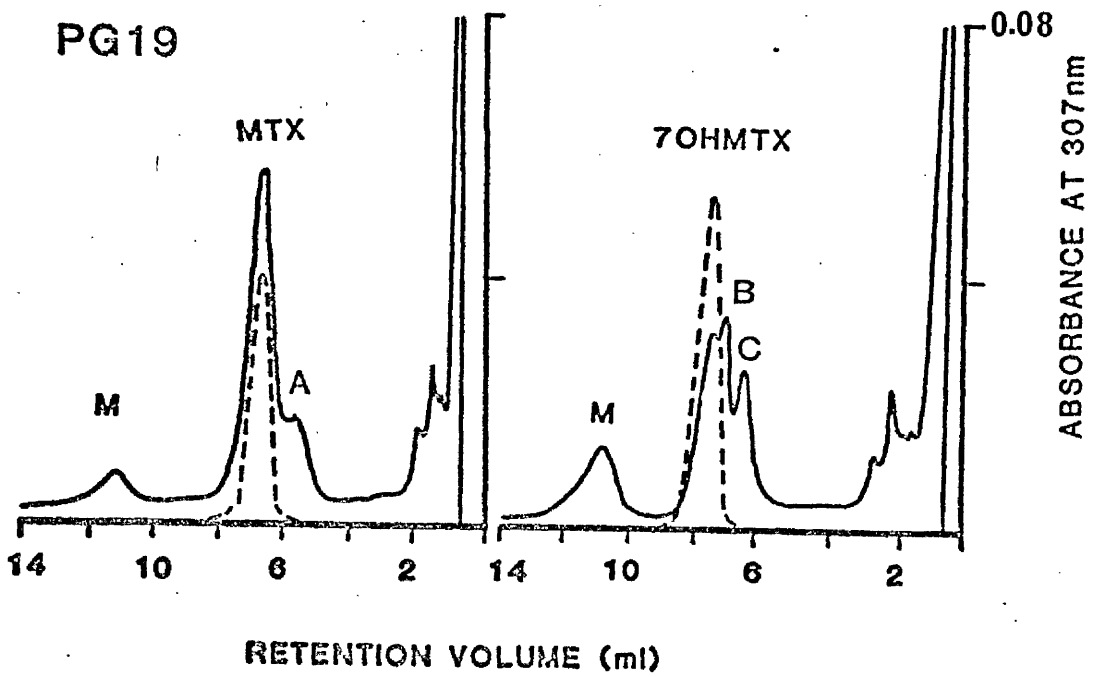


FIGURE 35a HPLC profiles obtained from culture medium 11 days after incubation of PG19 cells in the presence of 10^{-6}M MTX or 7-OHMTX.

M = substance present in medium

A,B,C = unknown metabolites

Position of MTX and 7-OHMTX shown dotted. Control cultures showed only peak M.

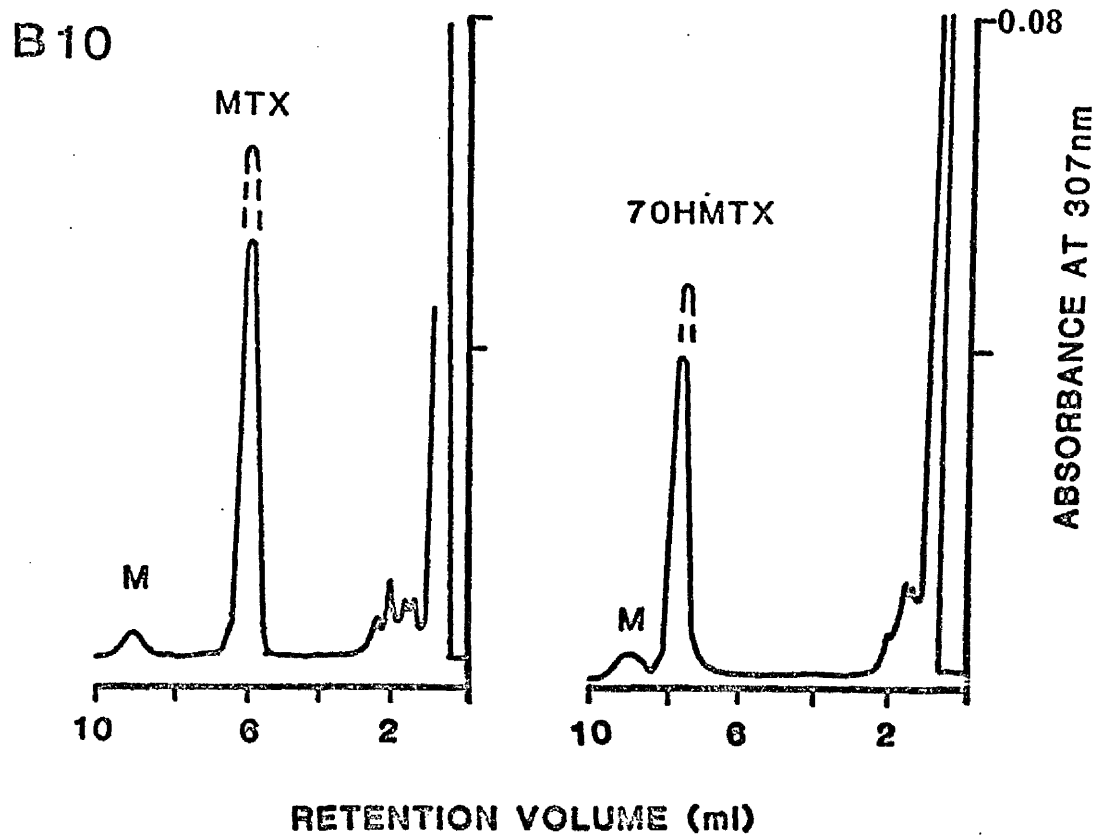


FIGURE 35b HPLC profiles obtained from culture media 11 days after incubation of B10 cells in the presence of 10^{-6}M MTX or 7-OHMTX.

M = substance present in medium

A,B,C = unknown metabolite

Position of MTX and 7-OHMTX shown dotted. Control cultures showed only peak M.

TABLE 22

Summary of HPLC Analyses of MTX and 7-OHMTX Metabolism in vitro by Different Cell Lines

LINE	MTX	7-OHMTX
Medium alone	No change	No change
Murine melanoma		
PG 19	Altered	Altered
Choriocarcinoma		
BeWo	No change	Altered
JaR	No change	No change
Human melanoma		
ADLER	No change	Altered
MYJ 15	No change	Altered
KOT	No change	Altered
B8	Altered	No change
B1O	No change	No change
MEL 57	No change	No change

2. Cell pellets

The pellet extracts injected onto HPLC showed no peak except the internal standard.

DISCUSSION

From the results of the bacterial experiments, we have confirmed that MTX passes into the cells in all species. No DAMPA or 7-OHMTX was detected either in the bacterial pellets or the supernatant broth. These studies therefore confirm that, at least in vitro, none of these 19 strains of gastrointestinal bacteria are able to deglutamate MTX in vitro.

In only one strain of E. Coli was a significant amount of a putative metabolite found in the pellet extract. There was insufficient of this preparation to allow definitive identification, however the 'metabolite' co-eluted with $MTXG_2$ and it is not inconceivable that this could be its true identity.

It has therefore been impossible to add to the observations of Levy and Goldman (1967) who reported the conversion of MTX to DAMPA by pseudomonas spp isolated from soil.

Using tracer MTX, Jolivet and Schilsky (1981) found MTX diglutamate and triglutamate in cultured human breast cancer cells. These metabolites were fractionated by HPLC and each fraction was counted by liquid scintillation counting. After fractionation of the same sample using Sephadex G15, they identified only the diglutamate and not the triglutamate. Using this HPLC method and UV detection, $MTXG_2$ was not detected in the cell pellets which were studied. It is probable that, with the small

numbers of cells available, the sensitivity of the HPLC/UV method is insufficient. However the failure to detect intracellular metabolites could be attributed to the conditions used since Rosenblatt et al (1978) reported that the accumulation of polyglutamate by the cells was dependent on the concentration of MTX in the culture medium, duration of incubation and stage of the culture cycle.

Studies on the culture broth in which human cell lines were incubated in the presence of MTX and 7-OHMTX produced different results, depending upon the cell lines employed. The PG19 (murine melanoma) strain is known to be MTX sensitive in vitro. This strain gave rise to changes in the pattern of MTX and 7-OHMTX in the culture medium, despite the fact that the majority of cells were killed within 48 hours of incubation. The peaks obtained, were again, not identified however one of the peaks in Figure 35a elutes at a retention volume identical to MTX in a culture to which only 7-OHMTX had been added, and raises the possibility that this line may be able to convert 7-OHMTX to MTX.

The B10 cells originate in a human melanoma cell line sensitive to MTX in vitro but resistant in vivo. It is of interest that these cultures showed only MTX or 7-OHMTX at the end of 11 days, with no other peaks in the chromatogram. These results were found to be reproducible in subsequent cultures of these cell lines.

One of the suggestions for the failure of MTX therapy *in vivo* against cells known to be sensitive *in vitro* is that the MTX is metabolised differently in these two environments. These studies show that cell-kill in vitro does not seem to be related to the 'metabolism' of MTX or its major metabolite.

The majority of cell lines studied alter 7-OHMTX as judged by HPLC analyses of supernatant broth. Less lines affect MTX itself. Once again there appears to be no relationship between susceptibility to the drug(s) and its removal from the culture medium. Detailed studies in MTX uptake and the effect of leucovorin-rescue in vitro on cell-kill by both MTX and 7-OHMTX have been reported elsewhere (Gaukrodger et al, 1983).

CHAPTER 7

DEVELOPMENT AND APPLICATIONS OF AN ASSAY FOR THE DETERMINATION OF ANTHRACYCLINES IN BIOLOGICAL FLUIDS

INTRODUCTION

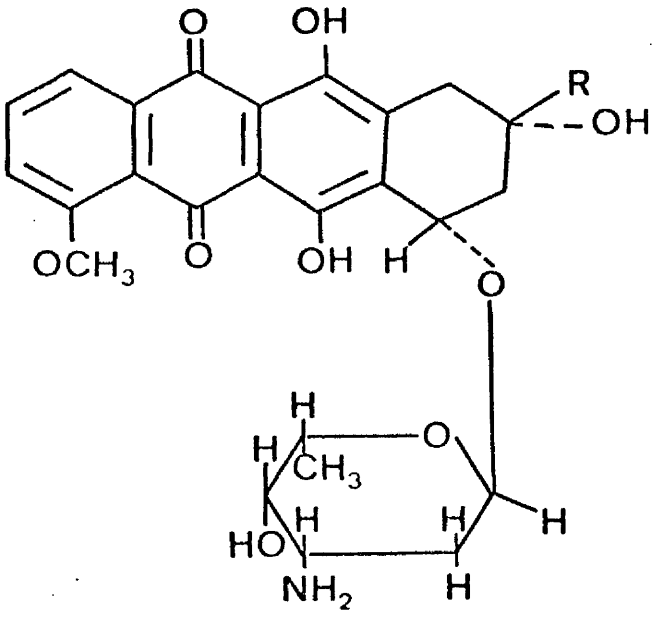
Structure

The anthracycline antibiotics doxorubicin, (adriamycin (ADR)) and daunorubicin (daunomycin (DAUN)) are important anti-neoplastic agents. The anthracyclines are derived from fermentation products of the fungus streptomyces peucetius (var. caesius).

The general structure of the anthracyclines is shown in Figure 36. Both compounds have tetracyclic ring structures with an unusual sugar, daunosamine, attached by a glycosidic linkage. ADR differs from DAUN by an hydroxyl group at C-14.

Mechanism of Action and Usage

The mechanism of action of the anthracyclines has been inferred from their ability to bind specifically to DNA by intercalation between adjacent base pairs, thus inhibiting DNA synthesis (Gabbay et al, 1976). Inhibition by ADR of DNA, RNA and protein synthesis has been documented in both cell-free systems and intact cells (Momparler et al, 1976). Furthermore, a recent study has shown that ADR will inhibit DNA polymerase in vitro, both competitively and non-competitively, and that the amino-sugar is critical for the ionic binding of anthracyclines to single-stranded DNA (Goodman, Lee and Bachur, 1977).



	R
Doxorubicin	$-C(=O)-CH_2OH$
Daunorubicin	$-C(=O)-CH_3$

FIGURE 36 The structures of Doxorubicin (ADR) and Daunorubicin (DAUN).

ADR is used against both primary and metastatic tumours (Gottlieb and Hill, 1974; Haskell, Eilber and Morton, 1975; Kraybill et al, 1977) while the clinical usefulness of DAUN is principally confined to the treatment of acute leukaemia in adults.

Dosage

The currently recommended dose of ADR is 60-75 mg/m^2 administered as a single rapid intravenous infusion and repeated after 21 days. The drug has also been given in doses of 0.5-1 mg/kg daily for 2-6 days or in doses of 20-30 mg/m^2 daily for 3 days or once weekly.

The recommended dose for DAUN is 30-60 mg/m^2 daily for 3 days or once weekly. The drug has also been given in doses of 0.8-1 mg/kg daily for 3-6 days (Calabresi and Parks, 1980).

Toxic Effects and Side Effects

The main toxic effects of ADR are as follows:

1. Cardiotoxicity

The most comprehensive information was collected in clinical studies of 1273 patients in a trial of ADR treatment carried out in 12 European Cancer Centres (Praga et al, 1979). The mean total dose of ADR was 268 mg/m^2 (range 15-1251 mg/m^2). The significant occurrence of cardiotoxicity is related to the total dose of ADR. There is an additional incidence of toxicity when vincristine is given either before, or concurrently, with ADR and with therapeutic regimes in which bleomycin is

given before ADR. Radiotherapy to the mediastinum, when given along with ADR, also gives rise to an increase in the incidence of cardiotoxicity. None of the 182 patients receiving ADR by slow infusion developed such toxicity. This was confirmed by Legha et al (1982).

Although the heart failure can be controlled by conventional treatment, death from side effects has been reported in 33-75% of patients who developed it (Lenaz and Page, 1976; Lefrak et al, 1973; Minow et al, 1977). A clear relationship exists between the cumulative dose of either drug and the incidence of congestive heart failure. Below a cumulative dose of 550 mg/m^2 of ADR , the incidence of congestive heart failure is less than 1% (Lenaz and Page, 1976; Lefrak et al, 1973). The incidence rises sharply with doses above 550 mg/m^2 affecting 30% of patients so treated (Lefrak et al, 1973; Lenaz and Page, 1976). Couch, Loh, Sugino (1981) reported sudden cardiac death of an 18-year old man following ADR therapy (8 months after the last dose of ADR).

2. Bone marrow depression

The depression of bone marrow function is associated with myelosuppression (Benjamin, Wiernik and Bachur, 1974). Thrombocytopaenia can also be troublesome particularly in patients who have received prior

radiation to the marrow, have bone marrow metastases, or who have compromised hepatic function (Benjamin et al, 1975).

Kelsen and Yagoda (1980) reported that the platelet count decreases before the white blood cell count and both reach nadir within 7-14 days, but usually return to pretreatment levels within 21 days after initiation of therapy.

3. Stomatitis

The most common toxic effect on the mucosa is stomatitis. Oral ulceration and occasional oesophageal lesions are seen with ADR administration, this is also dose-dependent (Kelsen and Yagoda, 1980). Lesser side effects are vomiting, nausea, alopecia, fever, chills, flushing and drowsiness (Benjamin et al, 1975; Kelsen and Yagoda, 1980).

Metabolism of Doxorubicin

The first step in the metabolism of ADR is carboxyl reduction at the C-14 position to doxorubicinol (ADRol). The small structural change does not prevent ADRol from binding to DNA and therefore this metabolite is therapeutically active (Bachur, 1976). The enzyme responsible is thought to be an aldo-keto reductase which has a ubiquitous distribution throughout the body tissue (Bachur, 1976).

There is considerable between-species variation in the proportion of parent drug metabolised to ADRol. Man is intermediate (Glode et al, 1977) between the highest reported rate which is found in the rabbit (Bachur, Hildebrand and Jaenke, 1974) and the lowest reported rate which is found in the rat (Tavoloni and Guarino, 1980). The further biotransformation of ADR and ADRol has principally been determined by Takanashi and Bachur (1976) as shown in Figure 37.

Cleavage of both ADR and ADRol at the glycosidic bond results in formation of the respective aglycones. Both are relatively insoluble in water and require conjugation to either sulphate or glucuronic acid before excretion. Both of these conjugated metabolites have been identified in rabbit and human bile (Bachur et al, 1974; Riggs et al, 1977). Further degradation includes demethylation and a variety of other metabolites have been identified in urine (Takanashi and Bachur, 1976) using thin-layer chromatography.

The aglycone and conjugated metabolites are therapeutically inactive but their contributions to the toxicity of ADR is unknown.

Methods of Assay

ADR and its metabolites have been determined by several different assay methods.

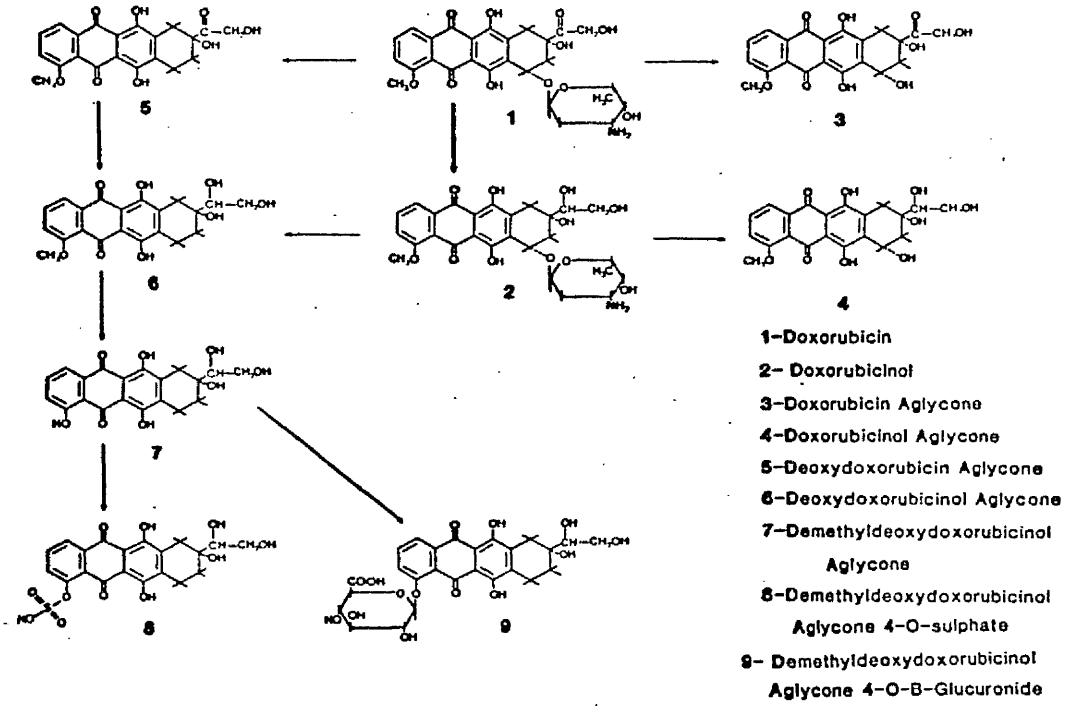


FIGURE 37 The metabolic pathway for Doxorubicin

1. Fluorimetric

Huffman, Benjamin and Bachur (1972) reported a fluorimetric method for the measurement of total ADR. The serum was mixed with 75% ethanol - 0.45 N hydrochloric acid and the resulting fluorescence was measured directly. This method is not specific due to cross-reaction by both metabolites and endogenous fluorophores.

2. Radioimmunoassay

Vunakis et al (1974) developed a radioimmunoassay for the measurement of ADR. Antibodies have been produced in rabbits, monkeys and goat by immunization with ADR-protein conjugation. The sensitivity of the method was 2 pmols/ml (1 ng/ml). It is somewhat non-specific due to interference by ADR aglycone (ADRone) (25%).

3. Thin-layer chromatography

Takanashi and Bachur (1976) reported a TLC method for the separation of ADR and its metabolites from urine. Quantitation was achieved by elution of the relevant areas and measurement of fluorescence or by the use of fluorescent scanners (Watson and Chan, 1976).

The TLC method is simple, rapid, and can give quantitative results. Until 1980, the majority of clinical studies have relied on this method for the analyses. However,the efficiency of chromatography in published reports is such that overlap of active and inactive metabolites occurs. There is now also a fair

amount of evidence that many of the metabolites are a result of degradation of ADR and its metabolites on the TLC plates (Poochikian, Cradock and Flora, 1981).

4. High performance liquid chromatography (HPLC)

Several methods have been published. All used a solvent extraction procedure. Solvents used include chloroform:isopropanol (Andrews et al, 1980; Pierce and Jatlow, 1979) and chloroform:methanol (Baurain et al, 1979; Strauss et al, 1980; Shinozawa, 1980).

Sepaniak and Yeung (1980) reported an HPLC method employing direct injection of urine samples. The method of Barth and Conner (1977) was applied only to pharmaceutical preparations and no extraction procedure was employed.

Normal-phase columns were used by Barth and Conner (1977), Baurain et al (1979) and Shinozawa (1980). Reverse-phase columns have been used by Pierce and Jatlow (1979), Andrews et al (1980) and Sepaniak and Yeung (1980).

Most of the authors used fluorescence detectors. UV detection was found to be sufficiently sensitive for the analysis of pharmaceutical preparations (Barth and Conner, 1977). Reported recoveries of ADR ranged from 65-99.7%. The sensitivity of such methods is high, ranging from 1.5-25 pmol/ml (0.8-13.6 ug/l). None of the authors reported studies of the possible interference by other anti-neoplastic drugs. This is surprising in view

of the fact that ADR is seldom used alone in treatment regimes. A summary of the details of extraction solvents, mobile-phase, packing materials, recovery precision, sensitivity, linearity and drugs interference is shown in Table 23.

Aims

The aims of this study were:

1. To develop an accurate, rapid, precise and sensitive method for ADR and its metabolites.
2. To investigate the effects of ion-pair agents on the chromatography.
3. To apply the method to clinical samples.

TABLE 23

The Characteristics of Published HPLC Methods for ADR is shown in this Table with Comparison Values

	EXTRACTION SOLVENT	MOBILE-PHASE (PACKING MATERIAL)	RECOVERY	PRECISION	SENSITIVITY	DRUG INTERFERENCE	LINEARITY
BARTH and CONNER 1977	No extraction	3.8% sodium acetate in isopropanol (Silica)	-	-	0.8 ng/ml	-	-
BAURAIN et al 1979	Chloroform:Methanol	Chloroform, methanol glacial acetic acid 0.3 mM $MgCl_2$ (Silica)	99.7	3.1	-	-	-
ANDREWS 1979	Chloroform:Propanol	Ammonium formate: tetrahydrofuran (ODS - hypersil)	50-70%	-	25 pmol/ml	-	0.025-1 nmol/l
PIERCE and JATLOW 1979	Chloroform: Isopropanol	50% acetonitrile in 0.01M phosphoric acid (ODS 5 µ)	65-75%	-	1 ng/ml	-	0-100 ng/ml
STRAUSS et al 1980	Chloroform:Methanol	0.05M NaH_2PO_4: methanol (ODS 5 µ)	95%	-	2 ng/ml	-	-
SHINOZAWA 1980	Chloroform:Methanol	3.8% sodium acetate in isopropanol (Silica)	98% Serum 66-96% Tissue	-	-	-	-
SEPANIAK and YEUNG 1980	Direct Injection	50% acetonitrile in 0.01M phosphoric acid (5 µ ODS)	-	-	10 ng/ml	-	Up to 50 ug/ml

MATERIALS AND METHODS

Doxorubicin (ADR), Doxorubicinol (ADRol), Doxorubicin aglycone (ADRone) and Daunorubicin (DAUN) were a gift from Farmitalia (Milan, Italy). Pentanesulphonic acid, hexanesulphonic acid, heptanesulphonic acid, octanesulphonic acid, tetraethylammonium bromide, and dodecyltrimethylammonium bromide were Fisons HPLC grade (Fison Scientific Apparatus, Longborough, England). Sodium dihydrogen phosphate was obtained from BDH. Solvents were HPLC grade and obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland). All other reagents were Analar grade and obtained from British Drug Houses (Poole, Dorset, England). Brij 35 was obtained from Technicon Chemical Co. (Hamilton Close, Basingstoke, Hants, England).

Apparatus

A Pye-Unicam HPLC system (Cambridge, England) consisting of an LC-XPS single piston reciprocating pump, a PM8251 single pen recorder and LC-FL detector with interference filters at 450 and 550 nm for the excitation and emission wavelengths respectively, were used.

The column (10 cm x 5 mm ID) (Shandon Southern Runcorn, England) was slurry packed with 5 u ODS Hypersil (Shandon Southern) using a Shandon column packer and fitted with a valve adaptor (Shandon Southern).

Manual injections were made via a Rheodyne 7125 injection valve (Scotlab Instrumentation Ltd., Law, by Carluke, Scotland).

Extraction Procedure Development

1. Choice of extraction solvents

One ml of serum spiked with 100 ug/l ADR, ADRol, ADRone and DAUN) was mixed with 1 ml of 10 mM phosphate-buffered saline (pH 7.8) (Shinozawa et al, 1980). The drug was extracted by shaking with 5 ml of mixture of chloroform:methanol (4:1 by volume) for 10 minutes. Due to emulsion formation, another 5 ml of organic-phase was added and shaken. After centrifugation, the upper aqueous layer was aspirated and 5 ml of the organic layer was transferred into a conical tube. The mixture was evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 100 ul methanol and 20 ul was injected onto HPLC column.

A second series of experiments was carried out using dichloromethane:isopropanol (4:1 v:v).

2. The effect of pH on the recovery of ADR and its metabolite

This experiment was carried out using 10 mM phosphate buffer or 10 mM phosphate-buffered saline over the pH range of 2-8.8. The extraction was carried out by using aqueous standard.

3. Choice of Internal Standard

DAUN has been used as internal standard by most workers (Pierce and Jatlow, 1979; Baurain et al, 1979). It has a similar structure and similar physiochemical properties to ADR and has not been used clinically as adjuvant with ADR. In chromatography, DAUN is well resolved from ADR and its metabolites. It was therefore used as internal standard in these studies.

4. Optimised Extraction Procedure

Serum and urine:

One ml of serum or urine was place in a Q + Q tube and mixed with 1 ml of 0.01M disodium hydrogen phosphate-buffered saline pH 7.8. To this was added 10 ml of dichloromethane:isopropanol (4:1) containing 200 ng of DAUN as internal standard. The mixture was shaken for 10 minutes in a lateral shaker (Griffin and George, London, UK) and centrifuged for 10 minutes at 2000 g. The aqueous layer was aspirated and the organic solvent was transferred to a conical tube and evaporated to dryness at 50°C under nitrogen.

The residue was dissolved in 50 ul of mobile-phase and 20 ul was injected onto the HPLC column. The ratio of analyte/internal standard peak areas were determined and compared with a calibration curve.

Initial Development of Liquid Chromatography Method

A 10 cm x 4 mm ID column containing 5 u ODS Hypersil was first tried with detection of the red ADR at its peak wavelength of 490 nm.

1. The eluting solvents methanol and acetonitrile (CH_3CN), alone, and a mixture of methanol and CH_3CN in proportion of 1:1 were investigated.
2. In further experiments octanesulphonic acid, an ion-pair, was added to the methanol:CH_3CN (50:50) at a concentration of 1 mM.
3. The addition of water or buffers to the mobile-phase in order to increase the polarity was investigated.

In all these experiments a mixture of 5 ug/ml each of ADR, ADRone, ADRol and DAUN was injected.

Further detection experiments were carried out using a fluorescence detector with interference filters at 450 and 550 nm for the excitation and emission wavelength respectively.

Further Development of the HPLC Method

1. The effect of increased solvent polarity

A solvent consisting of CH_3CN:10 mM phosphoric acid in proportion of 1:1 v:v was found to elute the ADR, metabolites and DAUN. Different proportions were used in order to determine the mobile-phase which yielded the highest resolution.

2. The effects of anionic surfactant on k' and resolution

To the appropriate solvent CH_3CN:10 mM phosphoric acid (35:65 v:v) were added increasing amounts of one of the following anions: pentanesulphonic acid, hexanesulphonic acid, heptanesulphonic acid or octanesulphonic acid. The concentrations used were 0, 0.75, 1.5, 3, 4, 5 or 6 mM.

Overnight washing with acetone was carried out between experiments to desorb the ionic surfactant from the reverse-phase packing materials.

Injection of the anthracycline mixture at the different ionic surfactant concentrations were made after several hours equilibration, and repeated until two successive results corresponded exactly, indicating that the column had achieved steady state.

3. The effects of cationic and neutral surfactants on the separation

To the appropriate mobile-phase were added increasing amounts of cationic and neutral surfactants. The cationic surfactants used were dodecyltrimethylammonium bromide and tetraethylammonium bromide. Polyoxyethylene lauryl ether (Brij) was used as neutral surfactant. The concentrations used were again 0.75-6 mM.

Linearity, recovery, precision, sensitivity and accuracy were calculated for each of the modifications of the method as follows:

Linearity

Linearity was studied by injecting 20 ul of the mixture of ADR, ADRone and ADRol and DAUN (IS) in methanol over the concentration range 0-10 ug on column (0-500 mg/l standard) ($0-10^{-3}$M).

Recovery

Sera of known ADR concentration to which IS had been added were assayed and the result was compared with direct injected standard mixture in order to calculate the absolute recovery.

Precision

Serum and urine:

The precision was assessed by determining ADR, ADRol and ADRone in serum (n = 20) at two concentrations 100 ug/l and 500 ug/l (20 ng, 100 ng/column) (2×10^{-7}M and 10^{-6}M).

Accuracy

Since there is a possibility of interference by a number of drugs, especially anti-cancer drugs, in samples obtained from patients.The response of the chromatographic method to directly injected aqueous solutions (1 g/l) of cyclophosphamide, dacarbazine, fluorouracil, vincristine, vindecine, vinblastine and cis-platinum, cytarabine, MTX, 7-OHMTX, DAMPA, trimethoprim, paracetamol and diazepam was checked. The last two were

included since they are commonly prescribed to patients receiving anti-cancer drugs. The accuracy was determined from recovery studies.

Sensitivity

The sensitivity of detection of ADR and each metabolite was determined by finding the lowest concentration in serum which would give rise to a peak with a height = 2 x baseline noise level.

Application of the Method to Biological Specimens

1. Human biological fluids

Two patients were given 40 mg ADR as an IV infusion over 4 hours. Blood samples were drawn from one patient at 0, 1, 2, 3, 4, 5 and 18 hours after commencement of infusion and at 0, 1, 4, 5 and 7 hours from the other.

Urine samples were collected every 2 hours, from -2 to 0 hours (start of infusion) 0-2 and 2-4 hours. One 12 hour sample was collected after completion of the infusion. The plasma samples and the urines were kept at -20°C until analysed.

2. Bacterial broth

A variety of bacterial strains were grown in Mueller-Hinton broth. These strains used were: Citrobacter Freundii, Klebsiella Pneumoniae, Pseudomonas Aeruginosa, Actinetobacter Calcoacetius Var Anitratrium, Escherichia Coli spp (E. Coli), Klebsiella spp and Pseudomonas spp. To the cultures in broth was added 10^{-4}M ADR and incubation continued for 24 hours. Blank

samples were obtained from cultures containing no ADR. At the end of the incubation the bacteria were removed by centrifugation at 1500 g to produce a clear supernatant solution which was stored at -20°C until analysed.

Method of Analysis

The HPLC method used to measure ADR and its metabolites as modified slightly as follows:

1. Human biological fluid

The mobile-phase used was acetonitrile:0.01M phosphoric acid (containing 6 mM Brij) in proportions 35:65 v:v.

2. Bacterial broth

The mobile-phase was acetonitrile:methanol:water containing (1 mM octanesulphonic acid) in proportions 30:40:30, v:v:v.

RESULTS

Extraction Procedure Development

1. Extraction with chloroform:methanol

This gave a low recovery for ADR (32%) and ADRol (60%). ADRone and DAUN were decomposed, two peaks appearing on the chromatogram as in Figure 38.

2. Extraction with dichloromethane:isopropanol

This gave a high recovery, and no decomposition occurred. This solvent was used in subsequent experiments.

3. Optimisation of extraction procedure

The effect of pH on the recovery of ADR, ADRol and ADRone extraction is shown in Figure 39 using DAUN as IS. From Figure 39 it can be seen that the most efficient extraction occurred at a pH of 7.8 using phosphate-buffered saline.

Development of Liquid Chromatography Method

1. ADR and its metabolites were unretained when eluted with methanol, acetonitrile alone and a mixture of both.
2. ADRone, ADRol, and ADR had the same k' which was different from DAUN. This occurred when 1 mM octanesulphonic acid was used as ion-pair with methanol:acetonitrile (50:50).

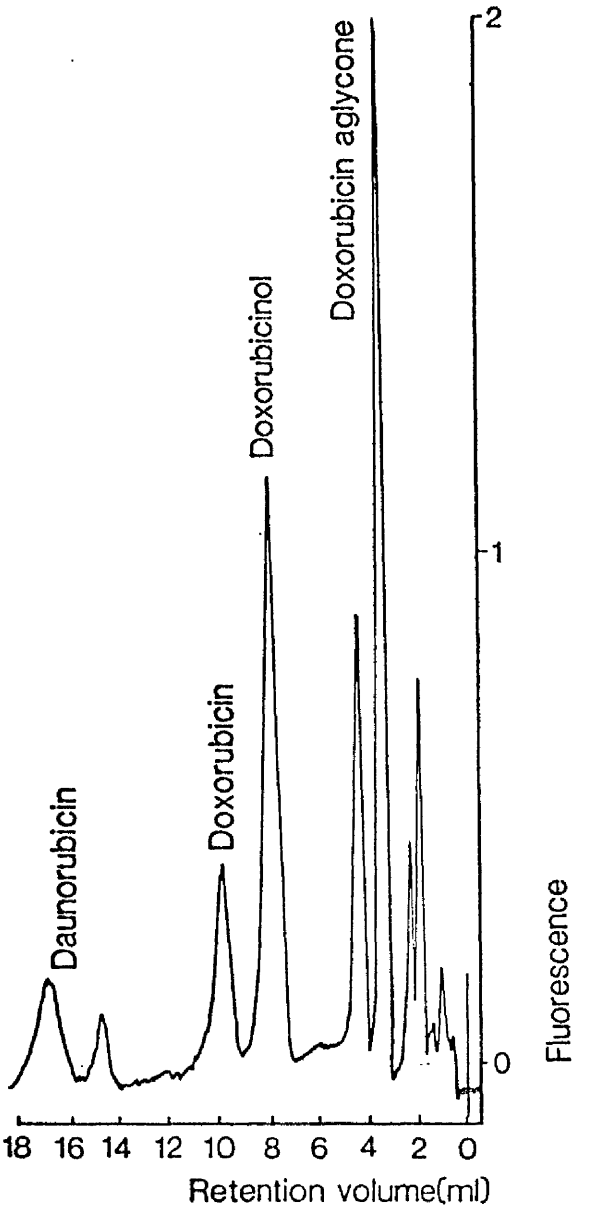


FIGURE 38 Chromatograms obtained from serum spiked with ADR and metabolites (DAUN as internal standard). Extraction with Chloroform:Methanol. ADRone and DAUN decomposed and appeared to be in two peaks.

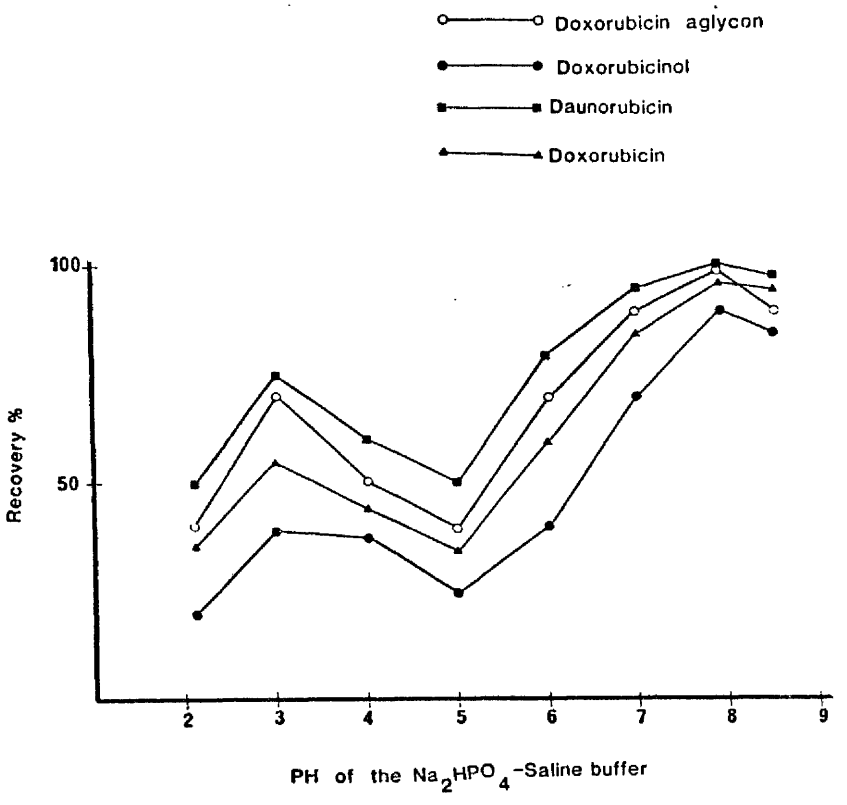


FIGURE 39 The effect of pH on the percentage recovery of ADR and its metabolites during extraction from serum.

3. The chromatogram of ADR and its metabolites with DAUN using methanol:water:acetonitrile (OSA%) in proportions of 40:30:30 (1 mM) as a mobile-phase is shown in Figure 40. The capacity ratios (k'), resolution and h value are shown in Table 24.

Further Development of the HPLC Method

1. The effect of increased solvent polarity

The effect of increasing polarity (by decreasing the concentration of CH_3CN in the final volume) on the retention time of ADR, ADRol, ADRone and DAUN is shown in Figure 41. Increasing the polarity increased the retention time but not by the same ratio for each component, i.e. the resolution was affected. Figure 41 shows the effect of increasing polarity on the resolution and indicates that the resolution between ADRone and ADRol decreased as the polarity increased.

The most efficient mobile-phase was found to be CH_3CN:10 mM phosphoric acid (35:65 v:v), this gave the best resolution. However, the retention time for DAUN was unacceptably long at 13.3 minutes.

2. The effect of anionic surfactants on k' and resolution

Table 25 shows the effect of increasing alkyl chain length of the anion on the k' of ADRone, ADRol, ADR and DAUN. The k' of ADRone alone was unaffected by increasing alkyl chain length.

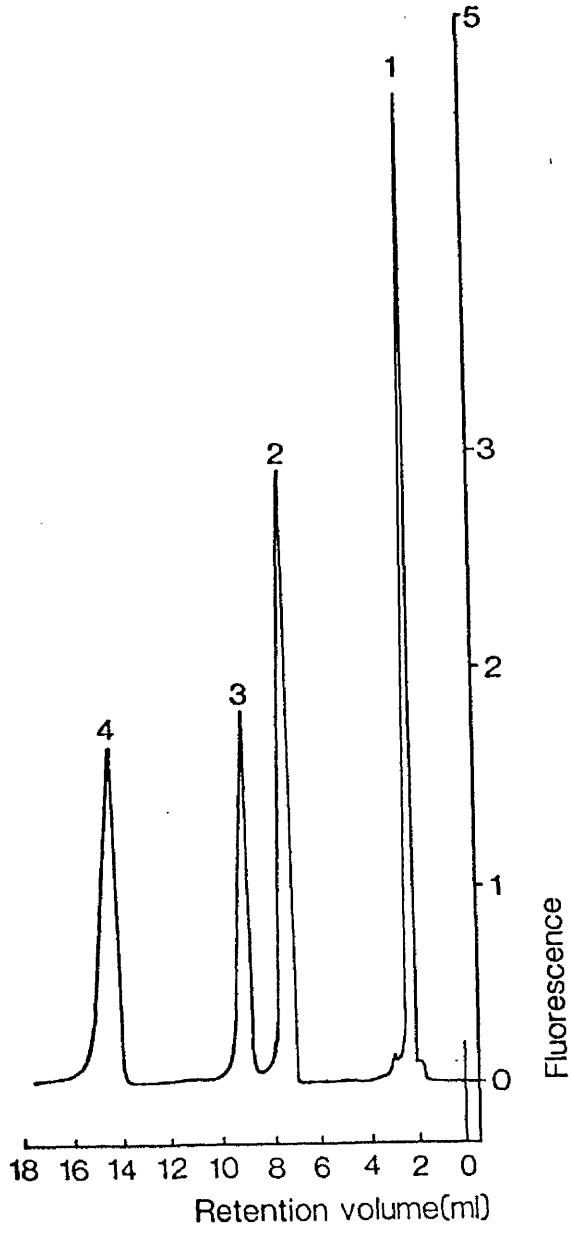


FIGURE 40 Chromatograms of ADR and metabolite standards with DAUN as internal standard. The mobile-phase was Methanol:Acetonitrile:Water containing 1 mM octane-sulphonic Acid (40:30:30).

Peak identities are:

1 = Doxorubicin aglycone (ADRone)

2 = Doxorubicinol (ADRol)

3 = Doxorubicin (ADR)

4 = Daunorubicin (DAUN)

TABLE 24

The Capacity Ratio (k'), reduced plate height (h) and the resolution (Rs) for ADR and Metabolites with DAUN as Internal Standard using CH_3CN:Methanol:Water containing 1 mM Octanesulphonic Acid in proportions 30:40:30 as Mobile-Phase.

	ADRone		ADRol		ADR	DAUN
k'	1.22		6.8		8.6	14.6
h	41		8.6		7.5	7.09
Rs		8.5		2	4.9	

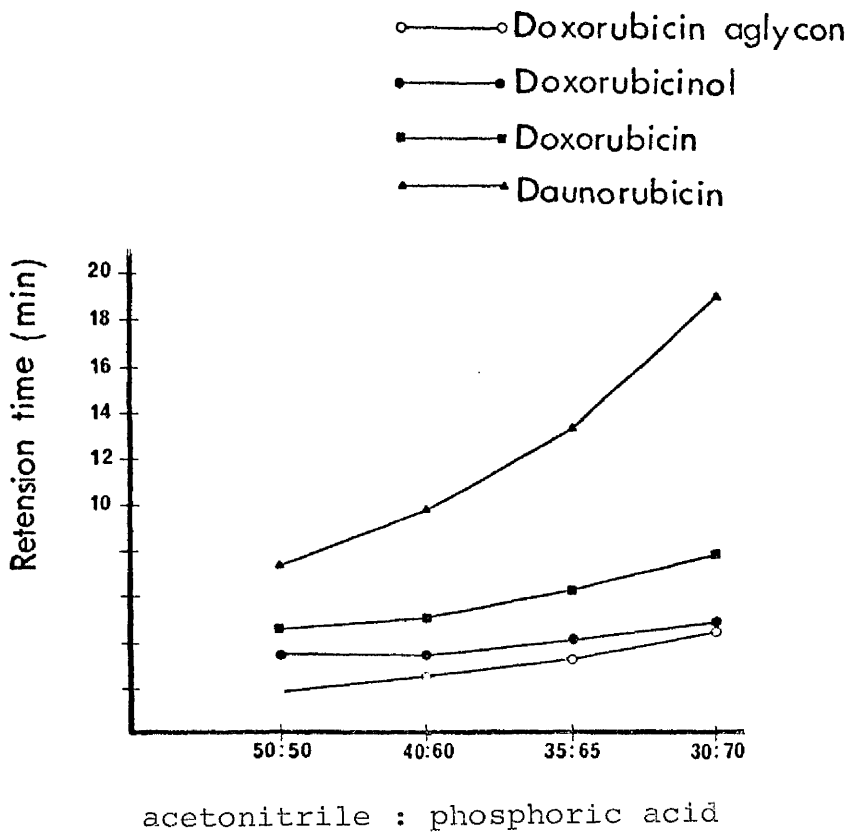


FIGURE 41 The effect of solvent polarity on the retention time of Doxorubicin aglycon, Doxorubicinol, Doxorubicin and Daunorubicin.

TABLE 25

Effect of Sulphonic Acid Chain Length and Concentration on the k' of ADRone, ADRol, ADR and DAUN

Surfactant Concentration	ADRone	ADRol	ADR	DAUN
Pentanesulphonic acid				
0	1.8	2.5	4.5	11
0.75	1.8	2.0	3.5	9
1.5	1.8	1.6	3.0	7.7
3.0	1.8	1.2	2.5	6.3
4.5	1.8	1.1	1.9	5.0
6.0	1.8	1.0	1.9	5.0
Hexanesulphonic acid				
0	1.8	2.5	4.5	11
0.75	1.8	2.0	2.4	8.7
1.5	1.8	1.6	2.9	7.4
3.0	1.8	1.3	2.5	6.0
4.5	1.8	1.1	2.2	5.6
6.0	1.8	1.0	2.0	5.0
Heptanesulphonic acid				
0	1.8	2.5	4.5	11
0.75	1.8	2.0	3.5	8.5
1.5	1.8	1.7	3.2	7.7
3.0	1.8	1.3	2.6	6.5
4.5	1.8	1.1	2.35	5.9
6.0	1.8	1.0	2.35	5.8
Octanesulphonic acid				
0	1.8	2.5	4.5	11
0.75	1.8	2.1	3.7	8.7
1.5	1.8	1.9	3.35	8.1
3.0	1.8	1.7	3.0	7.0
4.5	1.8	1.6	2.8	6.6
6.0	1.8	1.6	2.45	6.0

Figure 42 shows the effect of increasing alkyl chain length of anion on the resolution between ADRol and ADRone.

Figure 43 shows the resolution between ADR and ADRol with the addition of sulphonic acids of different chain length to the eluant. 3 mM pentanesulphonic acid was found to give the best resolution in association with low k' values.

3. The effect of cationic and neutral surfactants on separation

The effect of tetraethylammonium bromide, dodecyltrimethylammonium bromide and Brij on the capacity ratios for ADRone, ADRol, ADR and DAUN is shown in Figure 44a, b,and c respectively. It can be seen that the Brij gave the best resolution along with an acceptably low retention time for DAUN. The most satisfactory mobile-phase was thus determined as CH_3CN:10 mM phosphoric acid (35:65) containing 6 mM Brij.

Linearity

At each stage of development the HPLC method was found to be linear over the range 0-10 ng on column sample weight.

Recovery

The absolute recoveries for ADR, ADRol and ADRone are shown in Tables 26, 27 and 28 for the method at different stages of development. The recoveries relative to DAUN as IS were calculated to be 97.1$\pm$3.8% at all stages.

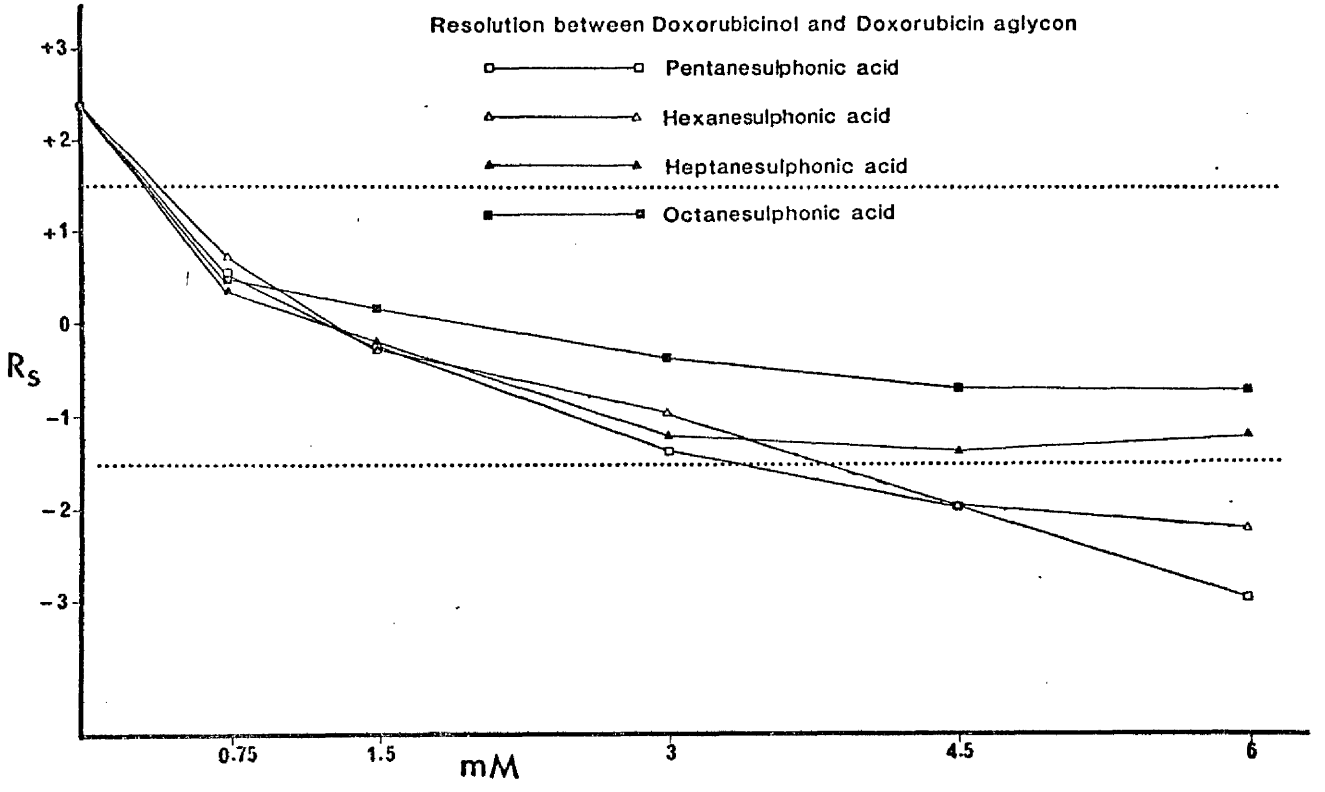


FIGURE 42 The effect of different concentrations of anionic surfactants on the resolution between Doxorubicinol and Doxorubicin aglycone. The area between the two dotted lines is that of incomplete resolution.

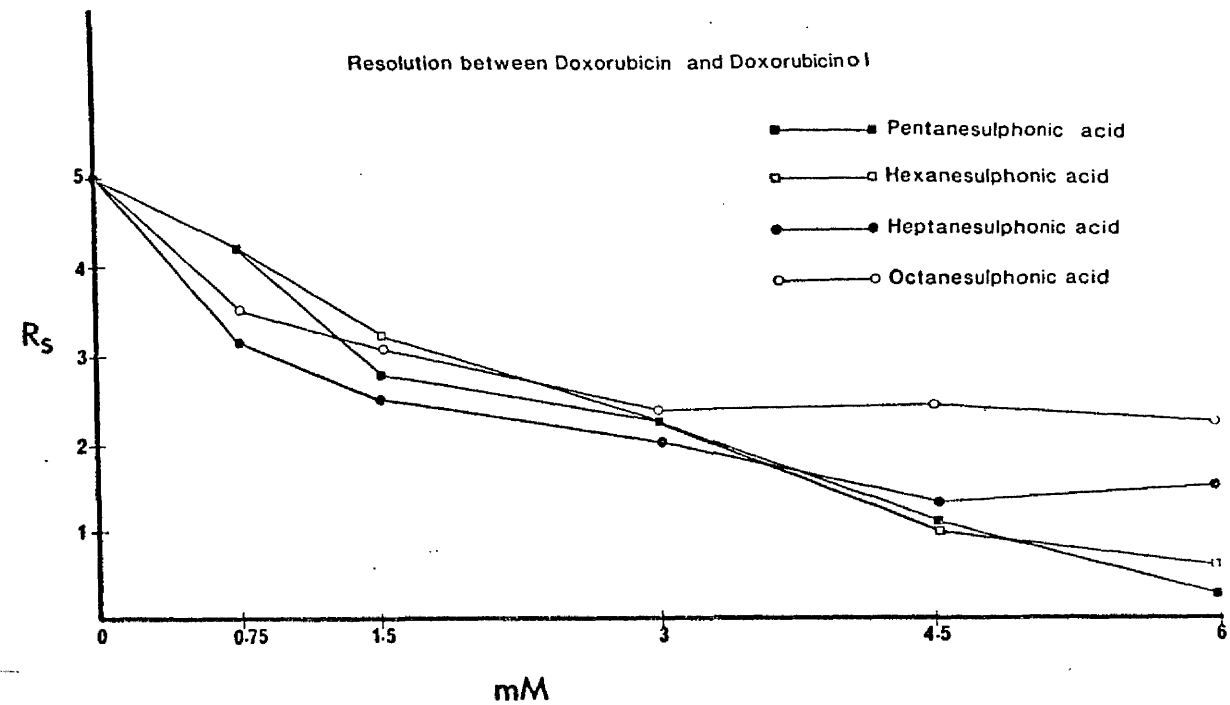


FIGURE 43 The effect of different concentrations of anionic surfactant on the resolution between Doxorubicin and Doxorubicinol.

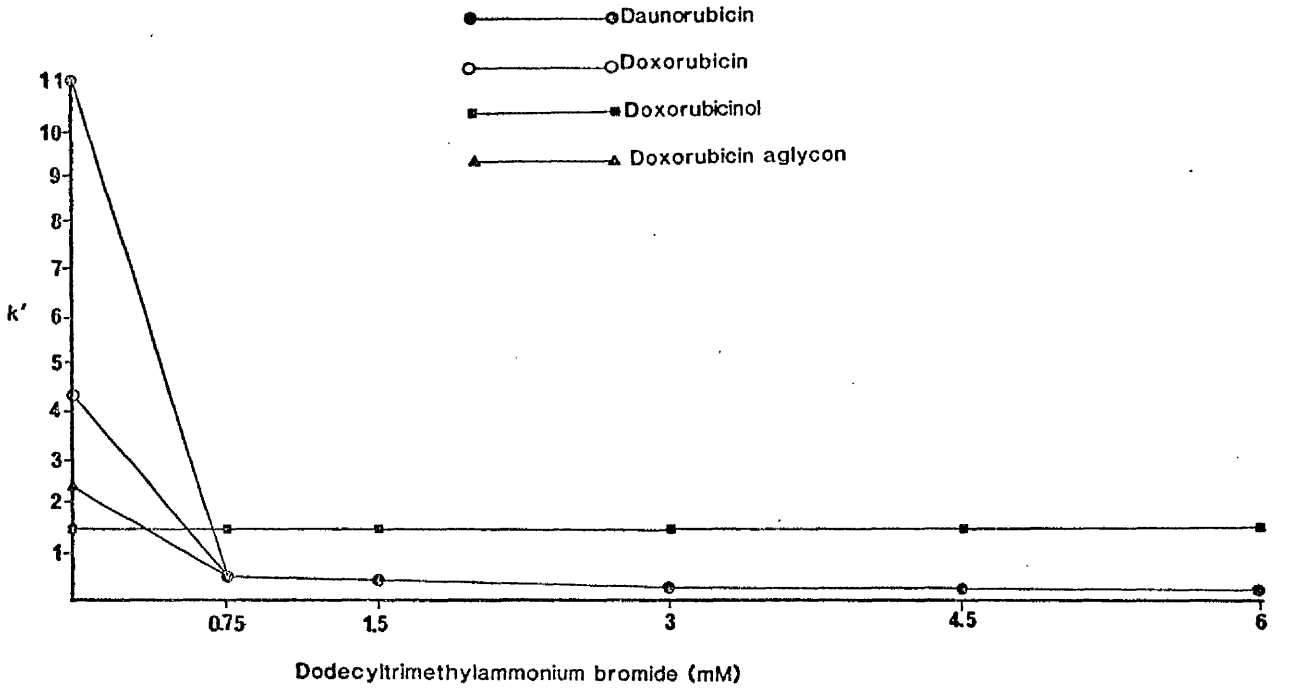


FIGURE 44a The effect of different concentrations of Dodecyltrimethylammonium bromide on the k' of ADR and its metabolites and DAUN.

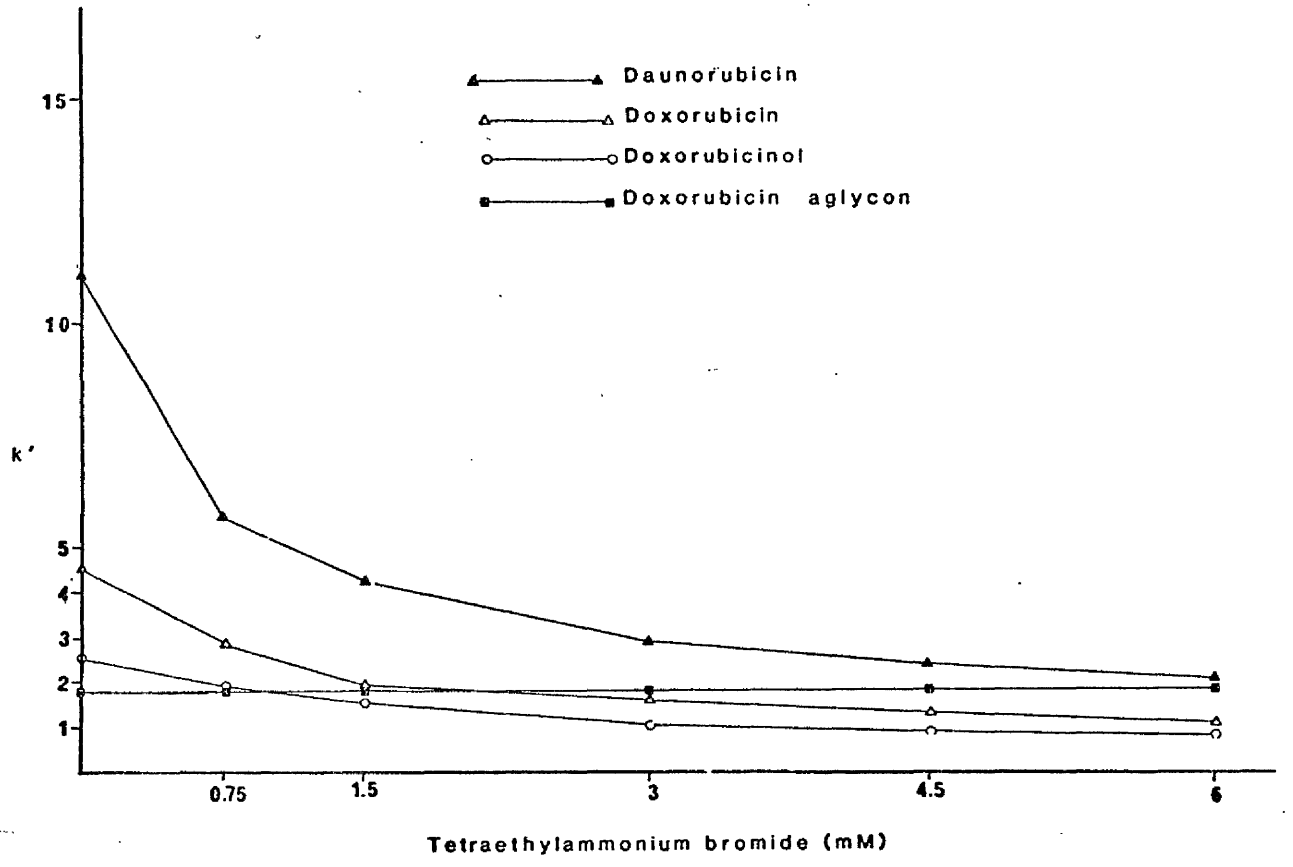


FIGURE 44b The effect of different concentrations of Tetraethylammonium bromide on the k' of ADR and its metabolites and DAUN.

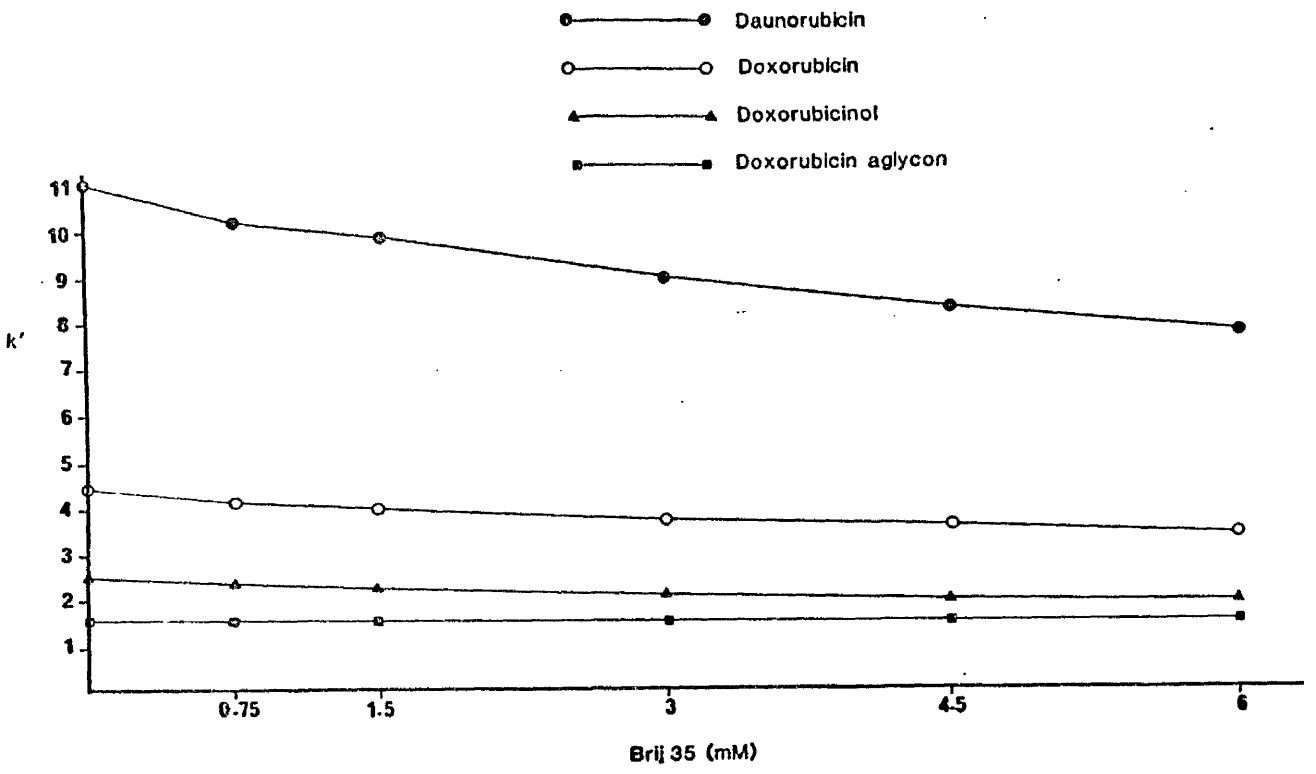


FIGURE 44c The effect of Polyoxyethylene lauryl ether (Brij) on the k' of ADR and its metabolites and DAUN.

TABLE 26

Absolute Recovery (n = 20) ADR and ADRol using Acetonitrile:Methanol: Water containing 1 mM Octanesulphonic acid in proportions 30:40:30 as a mobile-phase

Compound	Concentration Mass Unit	Molar Unit	Recovery
ADRol	10 ug/l	1.83×10^{-8}	90±5.3
ADR	10 ug/l	1.83×10^{-8}	73±2.7
ADRol	100 ug/l	1.83×10^{-7}	95.1±2.4
ADR	100 ug/l	1.84×10^{-7}	84.7±3.1

TABLE 27

Absolute Recovery (n = 20) of ADR and its Metabolite, using Acetonitrile:0.01M Phosphoric Acid containing 3 mM Pentanesulphonic Acid in proportions 35:65 as a Mobile-Phase

Compound	Concentration Mass Unit	Molar Unit	Recovery
ADRone	100 ug/l	2.4×10^{-7}	72.8±4.7
ADRol	100 ug/l	1.83×10^{-7}	87.5±7.7
ADR	100 ug/l	1.84×10^{-7}	82.75±6.1
ADRone	500 ug/l	1.2×10^{-6}	74.5±2.46
ADRol	500 ug/l	9.16×10^{-7}	96.2±3.6
ADR	500 ug/l	9.19×10^{-7}	97.3±2.3

TABLE 28

Absolute Recovery (n = 20) ADR and its Metabolites using Acetonitrile: 0.01 Phosphoric Acid containing 6 mM Polyoxyethylene Lauryl Ether (Brij) in proportions 35:65 as a Mobile-Phase

Compound	Concentration Mass Unit	Molar Unit	Recovery
ADRone	100 ug/l	2.4×10^{-7}	82.1±5.1
ADRol	100 ug/l	1.83×10^{-7}	92.2±4.7
ADR	100 ug/l	1.84×10^{-7}	95.4±3.9
ADRone	500 ug/l	1.2×10^{-6}	90.1±5.5
ADRol	500 ug/l	9.16×10^{-7}	92.4±4.8
ADR	500 ug/l	9.19×10^{-7}	95.2±3.6

Precision

The precision studies are summarised in Table 29. All coefficients of variation were below 10%.

Accuracy

None of the drugs examined interfered with the assay of ADR and its metabolites, nor were any endogenous peaks found which interfered with ADR and its metabolite peak

Sensitivity

The limit of sensitivity of the three methods using plasma extracts was found to be 1.5 ug/l (3 nmol/l).

Application of the Method to Biological Specimens

1. Human biological fluids

(a) Serum

A chromatogram obtained from an extract of pre-infusion serum is shown in Figure 45. Figure 46 shows the chromatogram obtained from an extract of serum from the same patient during treatment with 40 mg ADR by IV infusion over 4 hours. The chromatogram obtained from serum of the same patient, 1 hour after the end of IV infusion, is shown in Figure 47. The plasma profile of ADR and its metabolites during and after the IV infusion is shown in Figure 48.

(b) Urine

A chromatogram obtained from extracted urine from the same patient is shown in Figure 49.

TABLE 29

Precision Studies on ADR, ADRol and ADRone (n = 20) using Different Methods

Compound	Mass Unit	Molar Unit	Precision 1[a]	Precision 2[b]	Precision 3[c]
ADRone	100 ug/l	2.4×10^{-7}	-	6.4	6.3
ADRol	100 ug/l	1.83×10^{-7}	2.5	8.8	5.1
ADR	100 ug/l	1.84×10^{-7}	3.6	7.3	4.1

a = The mobile-phase acetonitrile: methanol:water (1 mM octanesulphonic acid)

b = The mobile-phase acetonitrile: 0.01M phosphoric acid (3 mM pentanesulphonic acid)

c = The mobile-phase acetonitrile: 0.01M phosphoric acid (6 mM Brij)

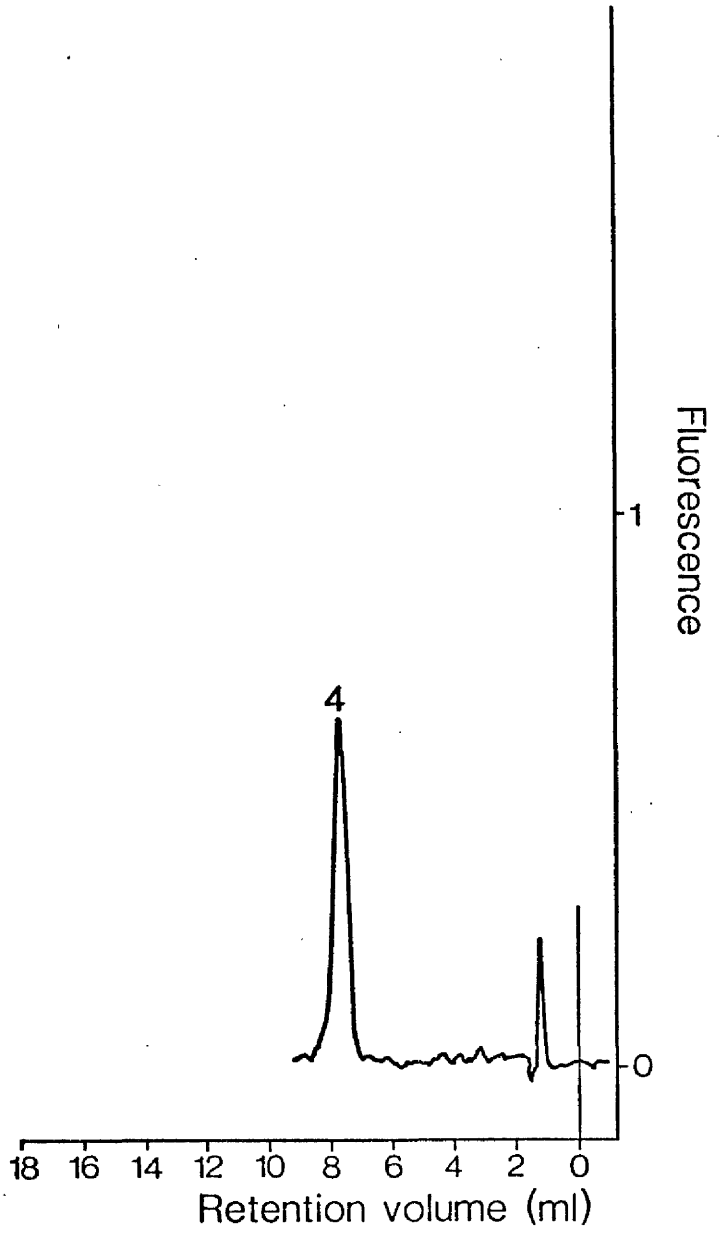


FIGURE 45 Chromatogram obtained from serum of a patient before an infusion of Doxorubicin.
4 = DAUN (internal standard).

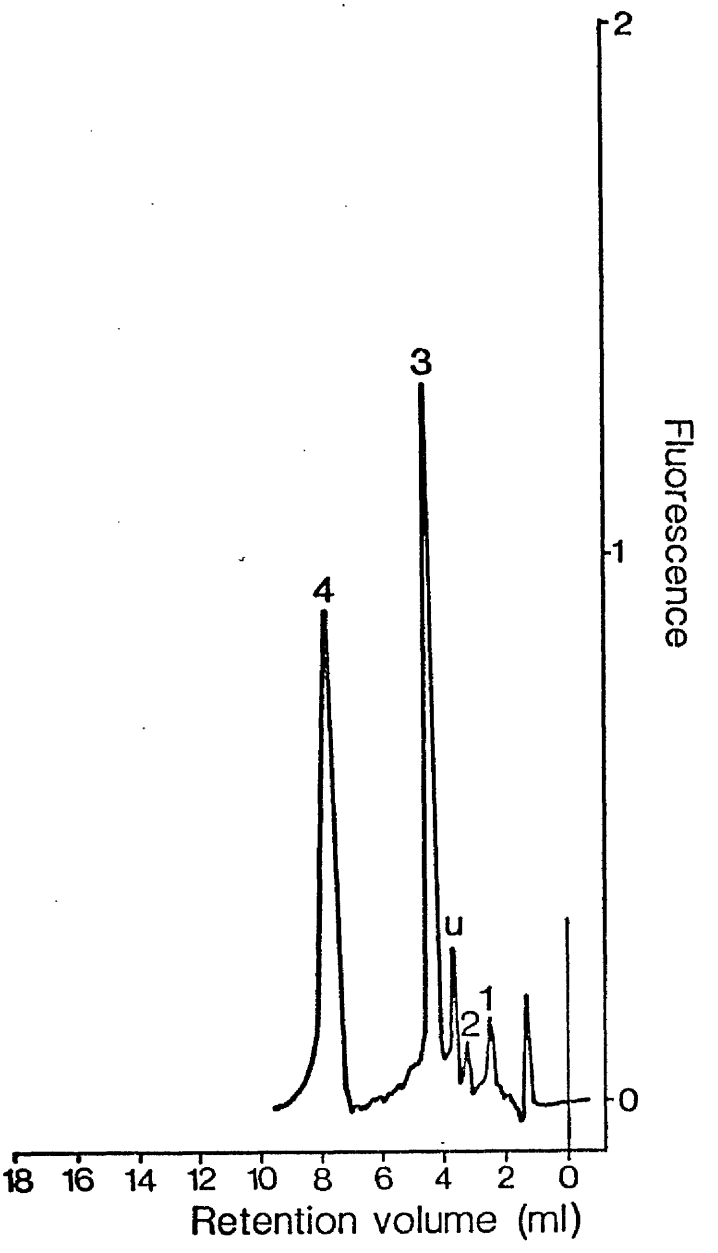


FIGURE 46 Chromatogram obtained from the serum of a patient treated with a 40 mg IV infusion of ADR over 4 hours. The sample was drawn at 3 hours (during the infusion).

Peak identities are as follows:

1 = Doxorubicin aglycone (ADRone)
2 = Doxorubicinol (ADRol)
3 = Doxorubicin (ADR)
4 = Daunorubicin (DAUN)
U = Unknown peak

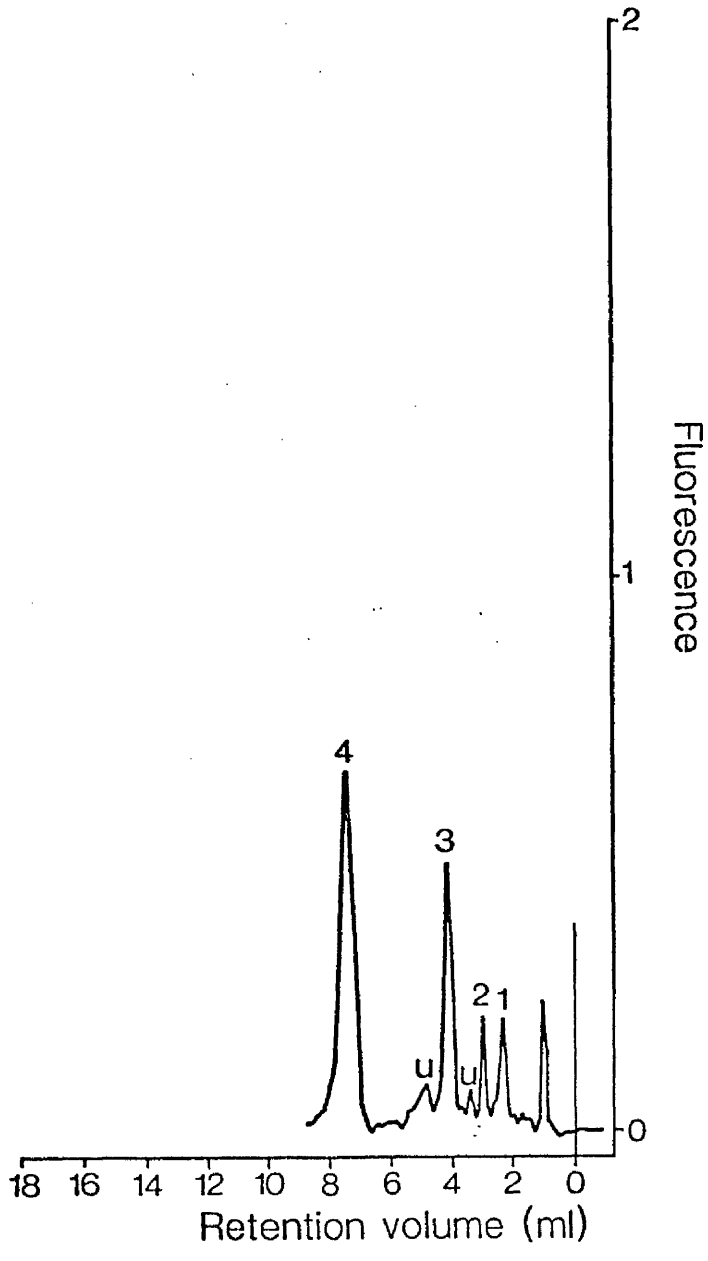


FIGURE 47 Chromatogram obtained from the serum of a patient 1 hour after stopping the infusion of ADR. Peak identities as in Figure 46.

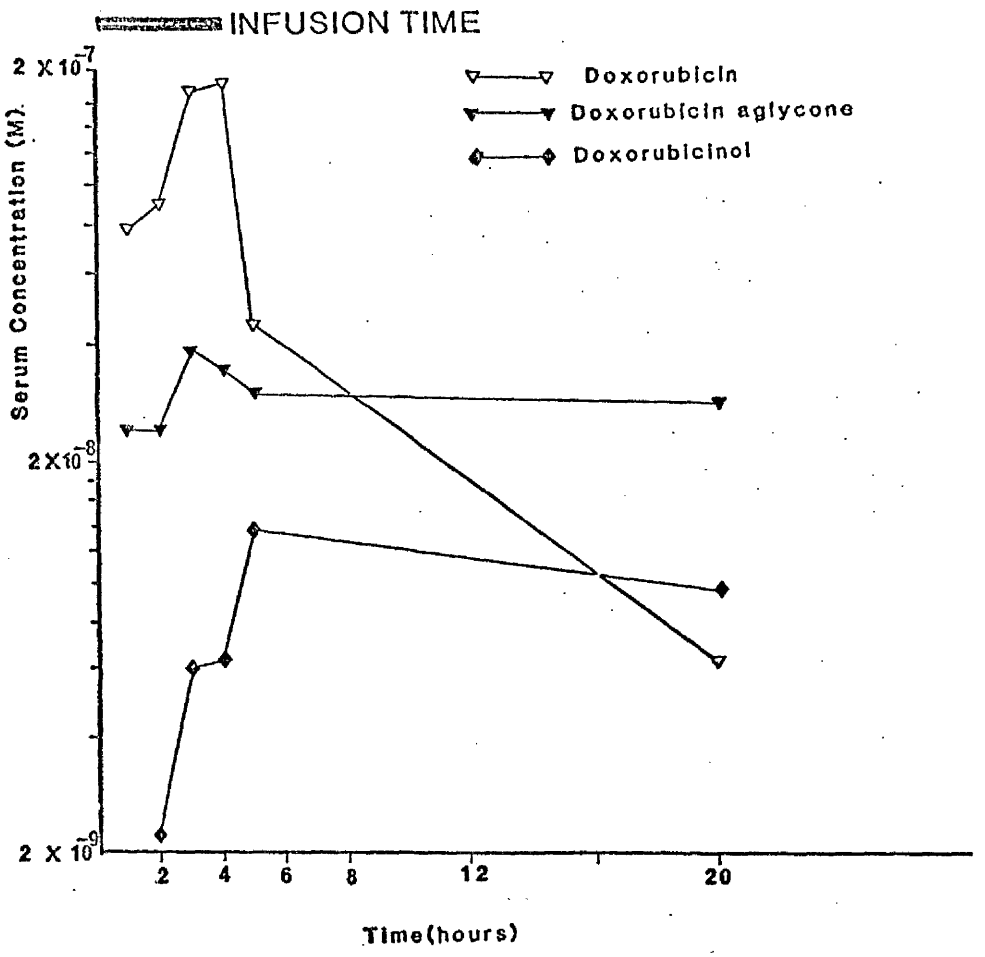


FIGURE 48 Plasma profiles of ADR and its metabolites during and after intravenous administration of ADR.

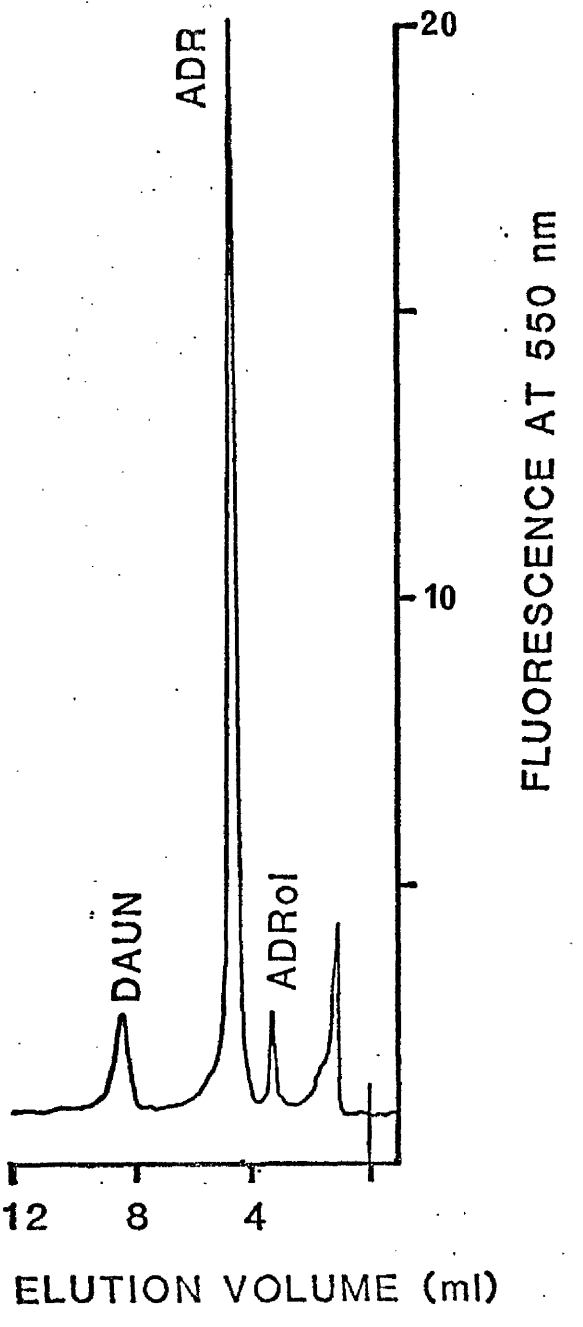


FIGURE 49 Chromatograms obtained from extracts of urine from a patient treated with 40 mg ADR as IV infusion over 4 hours.
Peak identities as in Figure 46.

The cumulative urinary excretion of ADR and ADRol from 0-28 hours is shown in Figure 50 for two patients. The total percentage of ADR excreted during 24 hours post infusion was about 6% of the total dose infused.

2. Bacterial broth

A chromatogram obtained from extracted supernatant broths is shown in Figure 51, showing the presence of an unidentified peak in the ADR containing broth. This peak was found to a different extent in all cultures (Table 30) and to a small extent (28%) in the broth incubated with ADR alone. It was not present in supernatant broth from cultures incubated in the absence of ADR. Although it has not been proven to be an ADR metabolite, it is highly suggestable that it is so, and its structure remains to be identified.

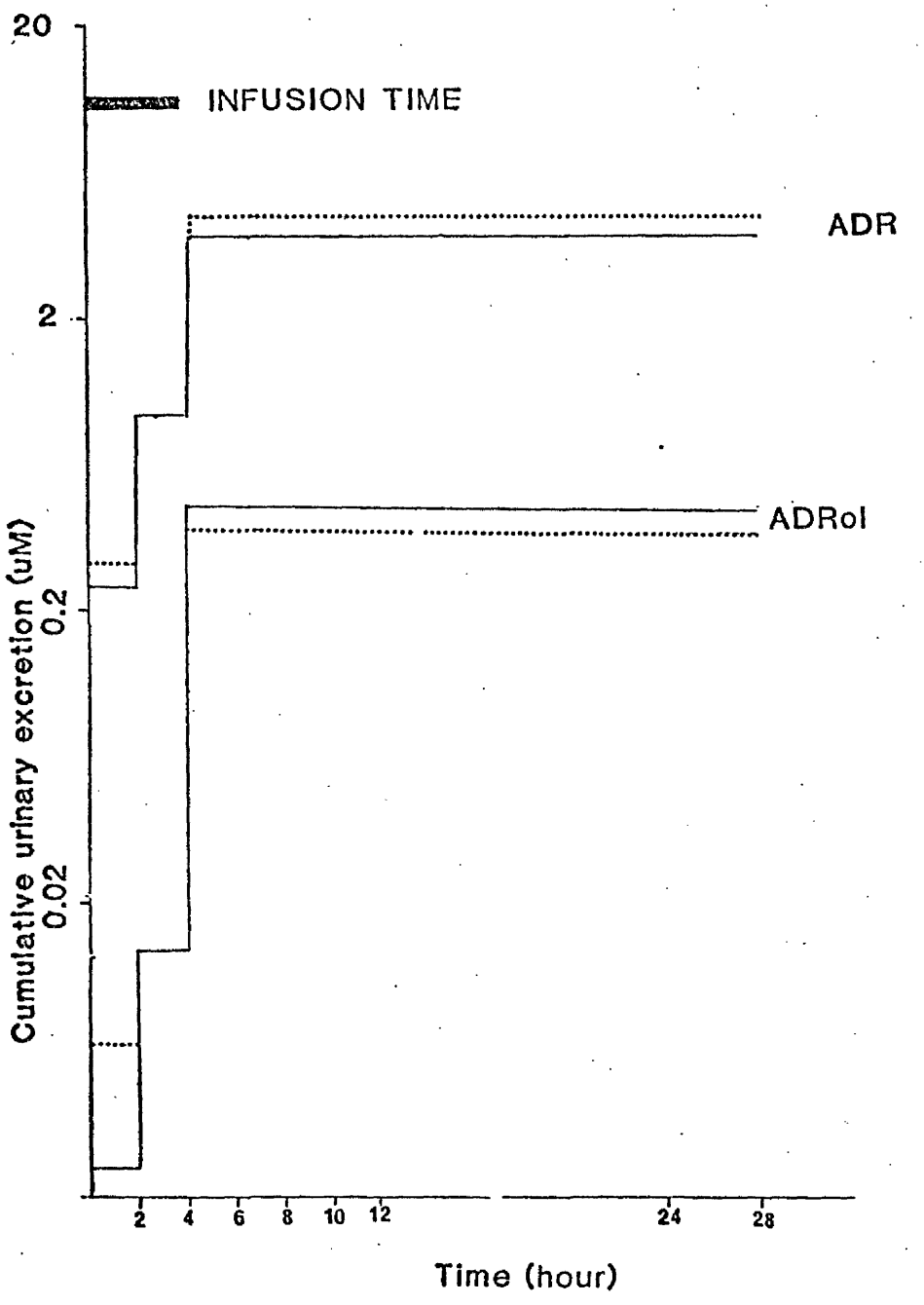


FIGURE 50 Recovery of ADR and metabolites from urine during and following ADR infusion. (Two patients)

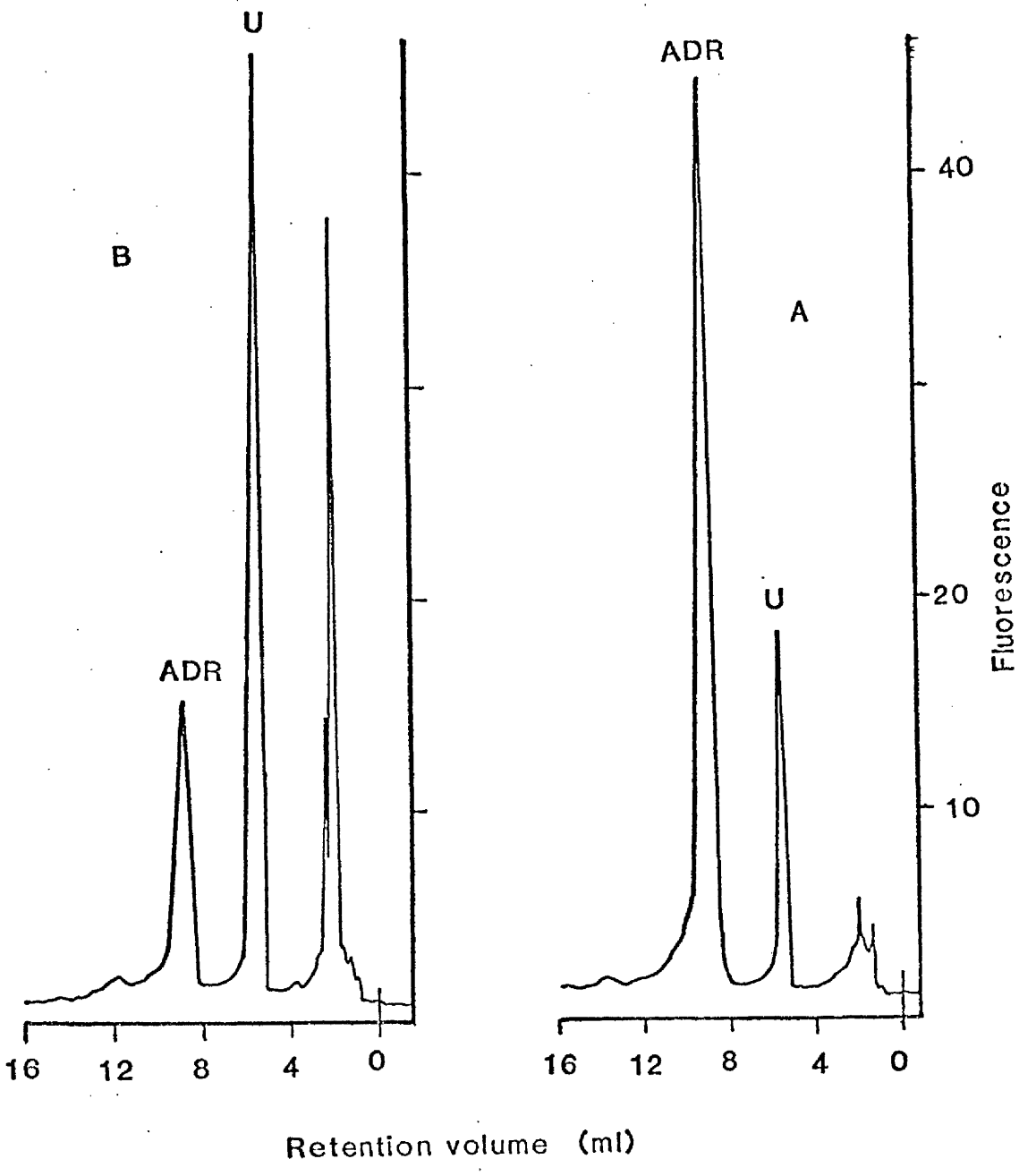


FIGURE 51 Chromatograms of ADR and unidentified peak found in:

A = Broth alone incubated with ADR

B = Bacterial broth incubated with ADR

Peak identities are:

U = Unidentified peak

ADR = Doxorubicin

TABLE 30

The Percentage of Unidentified Peak present in Cultures of Different Strains of Gastrointestinal Bacteria isolated from Different Patients

Bacterial SPP	% Concentration of* Unidentified Peak
Citrobacter Freundu	100
Klebsiella Pneumoniae	100
Pseudomonas Aeruginosa	77
Acinetobacter Calcoacetius var. Anitratrium	45
Escherichia Coli (E. Coli)	100
E. Coli	55
Klebsiella SPP	92
E. Coli	100
E. Coli	26
E. Coli	100
Pseudomonas SPP	40
Pseudomonas Aeruginosa	83
E. Coli	100
Klebsiella SPP	22
E. Coli	100
E. Coli	100
E. Coli (non-lactose fermenter)	100
E. Coli	100
Pseudomonas SPP	50
E. Coli	26
Broth	28

$$* \text{ Unidentified Peak\%} = \frac{\text{height of Unidentified peak}}{\text{height of ADR peak + height of unidentified peak}} \times 100$$

DISCUSSION

Method Development

ADRone and DAUN were decomposed by extraction with chloroform:methanol while dichloromethane:isopropanol extraction yielded a good recovery with no decomposition. This suggests that impurities in the chloroform are the most likely cause. The dichloromethane:isopropanol was found to be an acceptable solvent for extraction. This finding is in disagreement with Baurain et al (1979), Strauss et al (1980) and Shinozawa et al (1980) who all used chloroform:methanol as extraction solvent. From Figure 39 it can be seen that the recoveries for ADR and its metabolite with DAUN were excellent at pH 7.8 using phosphate buffered saline although the extraction efficiency was acceptable over a wide pH range (2-8.8).

The earlier method gave good resolution but showed a high retention time for DAUN. In addition the capacity ratio of ADRone was low at 1.22 and the peak co-eluted with endogenous components of plasma and urine.

When CH_3CN:phosphoric acid was used as mobile-phase ADR, its metabolites, and DAUN eluted from an ODS column with good resolution but with unacceptably high retention times.

This method was found to have acceptable sensitivity, accuracy, recovery and precision with the added advantage of fast pre-sample work up.

At a pH of 2.2, the major ionisation of the anthracyclines would appear to reside in the amino grouping of the sugar moeity which would be in the form of NH_3^+. The fact that the one metabolite which lacks this ionising grouping (ADRone) is unaffected by any of the surfactants, supports the view that any other ionisations on the tricyclic ring are insignificant in this chromatographic system.

The results obtained using the sulphonic acids exclude the possibility that ion-pairing is the only mechanism in force, since formation of the less polar ion-pair would lead to an increased k' for the three ionised species. This fact suggested that in this system the retention of the analytes is due not to affinity for the non-polar C_{18} chains but for free silanol groups which may carry a negative charge. The effect of the sulphonic acids may therefore be suppression of the ionisation of the weakly ionised silanol groups by competition for protons. The effect on k' of surfactant alkyl chain length may reflect the effects of polarity, increasing concentration also decreasing k'; since the proposed ion-exchange mechanism would be expected to be saturable, it is likely that a combination of suppression of ion-pair sites and modification of stationary-phase polarity is occurring.

The similar effects exhibited by the cationic surfactant molecules can be explained by competition between these positive charged ions and the cationic anthracyclines for the OH^- groupings on uncapped silanol groups.

The effect of the non-ionised Brij is unrelated to charge and is a less strong effect, probably due to the effect of the detergent on either solvent viscosity, although there were no discernable pressure changes, or surface tension effects at the interface between the solvent and the surface of the packing material. It may be that such surfactant effects also make some contribution to the reduction in k' resulting from the use of the cationic and anionic detergents.

In summary, an investigation into the effect of ion-pairing agents on the retention of anthracycline molecules on a reverse-phase column (Hypersil ODS 5 u) has revealed that the mechanism is not purely reverse-phase but may contain an element of ion-exchange chromatography involving uncapped silanol groups although this packing is claimed to have 'capped' silanol groups. An acceptable analytical method for the determination of anthracyclines at pH 2.2 using a non-ionic detergent has been developed using this packing material, but further studies particularly on other reverse-phase materials should be carried out to confirm this explanation.

Human Biological Fluids

The chromatogram obtained from the serum pre-infusion shows no interfering endogenous peaks. The sensitivity of the assay used was sufficient to detect less than 3 ng/column of the ADR metabolites. The unidentified peak found in each of the subsequent samples was either an impurity of the ADR pharmaceutical preparation (Barth and Conner, 1977) or one of the 8 metabolites which were detected by Andrews et al (1980). The height of the unidentified peak was less in Figure 47 than Figure 46 which suggests that it is most probably an ADR impurity.

The formation of the active metabolite (ADRol) is shown clearly in Figure 48. The concentration of ADRol was greater than that of ADR at 20 hours after the start of the infusion. The concentration of ADRone was also greater than that of ADR at 20 hours post infusion. ADRone has an almost flat elimination curve which indicates the difficulty in the excretion of this non-polar metabolite. The low total renal excretion ($\approx$6%) obtained from both patients suggests that the tissue pool for ADR is large, and that non-renal routes of excretion may well predominate.

The data obtained in this study are insufficient for pharmacokinetic calculations but are good enough to show that the method described can be applied to human

biological fluids and offer a method of studying both ADR and its two major metabolites in order to further elucidate the way in which this drug is handled in man.

Bacterial Broth

The studies using bacteria encountered one of the problems which have given difficulties to most others who have studied ADR metabolism, namely the breakdown of this rather unstable molecule during incubations and/or analysis.

ADR incubated in nutrient broth gave rise to a product which was present consistently at a peak height of 28% relative to that of the remaining ADR.

Incubation of ADR in broth in the presence of bacteria gave rise to widely different ratios, but no other peaks in the chromatogram.

Table 30 shows that within the limits of experimental error, 8 species gave rise to a pattern similar to that of the broth alone i.e. a 'metabolite' peak of relative height 22-50%. Ten species produced a metabolite peak considerably greater with consequent reduction in the amount of ADR recovery.

This in itself does not prove any bacterial metabolism, since the degradation of ADR may be related to the other factors such as alteration in pH or catalysis of breakdown by adsorption to bacterial cell walls.

These studies therefore show only that neither of the known in vivo metabolites which it was possible to measure quantitatively, were produced in vitro by a variety of bacterial strains isolated from human gut.

The fact that the peak was not detected in vivo also suggests that there is no detectable involvement of gastrointestinal bacteria in ADR metabolism in man, any metabolites which may be produced being excreted in the faeces rather than being absorbed.

The conclusion from these early studies are that the method developed is capable of measuring ADR and at least two of its metabolites in plasma and urine following the administration of doses in the range currently regarded as optimal. It remains to be seen whether the regular measurement of these analytes could lead to improvements either in the efficiency of treatment or in avoidance of toxicity. The bacterial studies indicate that it is unlikely that gastrointestinal bacteria contribute significantly to the variations in the effect of ADR in man.

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Methotrexate

The assay developed for MTX and its metabolites has several advantages over other currently available methods. MTX is labile at alkaline pH, thus ion suppression with alkali to allow liquid/liquid extraction is an unattractive approach due to the losses that may be incurred. Other workers have used protein precipitants (Nelson et al, 1973; Howell et al, 1980), but the studies conducted in this thesis indicate that the high losses and potential for interference make such assays sub-optimal. Donehower et al (1979) used a liquid/solid extraction. By refining and optimising this technique it has proved possible to obtain reproducibly high recoveries of MTX and its important Phase I metabolites; this has ensured the assay is robust in use and is very sensitive for all these components. It proved possible to inject urine directly onto the HPLC column, this ensured complete recovery whereas Donehower et al (1979) suffered losses of MTX and metabolites using a liquid/solid extraction due to urinary salt competition with the analytes and the ion-exchanger.

The use of an ion-pairing agent in the HPLC eluant has afforded excellent chromatographic efficiency and effective resolution of MTX and its metabolites,

including the Polyglutamates $MTXG_1$ and $MTXG_2$. The use of phosphoric acid instead of a buffer removes the problem of salt crystallisation.

Using this chromatographic system and using heat precipitation the HPLC method was used to monitor liver homogenate synthesis of the 7-hydroxylated products of MTX and DAMPA. The identity of the 7-OHDAMPA product was confirmed by NMR and EI/MS, unfortunately it has proved impossible to obtain a definitive identification of the peak found in clinical samples that has a k' identical to 7-OHDAMPA. However, there is some evidence to support the view that this is also 7-OHDAMPA; the UV spectrum of the two peaks were superimposable, also the peak is only observed in urine samples containing DAMPA. This study also supports the view that 7-OHDAMPA in vivo is a result of hepatic hydroxylation of DAMPA rather than deglutamation of 7-OHMTX which in turn adds further evidence against DAMPA being a product of enterobacterial metabolism as proposed by Donehower et al(1979). These findings and the plasma DAMPA concentration time curve tend to confirm the report of Chatterji et al (1978) that the source of DAMPA is the IV infusion.

The clinical significance of DAMPA and 7-OHDAMPA is unknown although their concentrations are two and three orders of magnitude respectively less than MTX. However, their solubilities in acid urine are markedly less than

for MTX (Donehower 1980) and they may contribute to nephrotoxicity, especially following prolonged administration of MTX.

The studies on high and low-dose MTX showed great variation in the pharmacokinetics within the population. Intra-individual variation in clearance was remarkably constant in the low-dose study, in the high-dose study this was not the case and may be due to the continuation of the disease process and adverse effects on the kidney.

The source of variation in bioavailability in the low-dose study has been ascribed to intra-individual variation in hepatic clearance on MTX presented via the portal vein, the so-called 'first pass' effect. Whether this is the result of differences in hepatic blood flow, enzyme induction by e.g. smoking or variation in gut absorption cannot be ascertained; this aspect merits further investigation.

It is of interest that in the high-dose kinetics there was no appreciable change in hepatic clearance of MTX on consecutive doses, the changes being mainly renal. There was a slight change in MTX AUC but a much more marked change in 7-OHMTX AUC and clearance. In view of the constancy of MTX hepatic clearance this change cannot be ascribed to hepatic enzyme induction and is therefore the result of an accumulation of 7-OHMTX which is not

being excreted renally; the impaired mechanism may be pH dependent solubility in the urine but this is unlikely, it is more probable that it is the combination of reduced filtration, and diminished secretion.

The investigations of MTX metabolism of bacterial and human cell lines in vitro provided evidence of some metabolic activity but there was no conclusive evidence as to whether such activity affected the toxicity of MTX to cells.

Anthracyclines

The use of fluorimetric HPLC for anthracycline drugs has resulted in the publication of a number of sensitive assays applicable to clinical material (Baurain et al, 1979; Andrews et al, 1979; Strauss et al 1980). Initial work showed that although the required separations could be achieved ADRone was poorly resolved from other components; it was noted that no other published method used an 'ion-pair' reagent. The experiments subsequently conducted resulted in the description of a novel ionic surfactant - solute - silanol interaction, and finally resulted in the use of an non-ionic surfactant. The principles described may be applied to many other analytes. The reduction in analysis times and enhanced resolution resulted in a highly sensitive assay.

The assay was applied to the study of the in vivo metabolism of ADR and the in vitro metabolism by bacteria. The former indicated the utility of the method

but lack of clinical material meant that studies on the role of metabolites in toxicity have yet to be fully developed. The in vitro work indicated that certain cells can metabolise ADR but the relevance of this to ADR cellular toxicity requires further investigation.

In conclusion the development and application of sensitive, accurate and precise assays of two anti-cancer agents, MTX and ADR, and their metabolites have enabled studies at high and low doses to be performed. With such assays it would be possible to monitor therapy and assess the effect of not only the parent drug but its metabolites. As such drugs have very low therapeutic indexes and are often used consecutively or concurrently there are very likely to be changes in their pharmacokinetics. Such changes could be of great clinical significance and could add to our ability to optimise chemotherapeutic regimes in cancer treatment. The assays described are capable of meeting such a challenge.

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