Journal of Chromatography, 337 (1985) 81—90
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2351

DETERMINATION OF SERUM METHOTREXATE AND 7-HYDROXYMETHOTREXATE CONCENTRATIONS

METHOD EVALUATION SHOWING ADVANTAGES OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

NELLIE SO, DAVID P. CHANDRA*, ION S. ALEXANDER

Children's Leukaemia and Cancer Research Unit, Prince of Wales Children's Hospital, High Street, Randwick, N.S.W. 2031 (Australia)

VALERIE J. WEBSTER

Department of Clinical Chemistry, Prince of Wales Hospital, Randwick, N.S.W. 2031 (Australia)

and

DARCY W. O'GORMAN HUGHES

School of Paediatrics, University of New South Wales, Sydney (Australia)

(First received April 12th, 1984; revised manuscript received August 23rd, 1984)

SUMMARY

The use of large doses of methotrexate (MTX), > 3 g/m^2, for the treatment of some malignant disorders requires careful monitoring of serum concentrations. A simple and sensitive method for the separation of MTX and 7-hydroxymethotrexate (7-OH-MTX) by reversed-phase high-performance liquid chromatography (HPLC) is described. The method involves deproteinizing the serum sample on a Sep-Pak C_{18} cartridge, followed by separation on a C_{18} column and detection at 313 nm. The extraction efficiency of free MTX from serum is 70% and the maximum sensitivity is $2.2 \cdot 10^{-8}$ *M*. A high degree of correlation was obtained between the HPLC method of serum MTX determination and an enzyme multiplied immunoassay technique. The HPLC method separates MTX from its analogues, or drugs which may be administered concomitantly with MTX. Concentrations of MTX and 7-OH-MTX achieved over a 24-h period during high-dose therapy, (500—1000 mg/m^2), and over 48 h for very-high-dose methotrexate therapy (8—12 g/m^2) are described. A significant observation is the presence of 7-OH-MTX in sera of patients 6 h after commencement of infusion.

0378-4347/85/$03.30

This method was also utilized for monitoring cerebrospinal fluid MTX concentrations.

INTRODUCTION

Methotrexate (MTX), amethopterin, 4-amino-N^{10}-methylpteroylglutamic acid, is a folate antagonist which acts by inhibiting intracellular dihydrofolate reductase. The consequence of this inhibition is the depletion of intracellular stores of reduced folates required for the 'de novo' synthesis of purines and pyrimidines, and for the metabolism of amino acids.

In combination with other antineoplastic agents, MTX is frequently used for the treatment of acute lymphoblastic leukemia, lymphomas, osteogenic sarcoma, and other forms of cancer. When using high doses of MTX, delayed excretion may lead to toxicity and therefore careful monitoring of serum MTX is important for optimal patient management [1]. In addition, the measurement of its less soluble major metabolite, 7-hydroxymethotrexate (7-OH-MTX) may be important, as it has been implicated in the development of renal toxicity [2].

Several methods have been reported for measuring concentrations of MTX in plasma and serum. These include enzyme inhibition [3, 4], protein binding [5], radioimmunoassay (RIA) [6, 7], fluorescence [8, 9], and high-performance liquid chromatography (HPLC) [10—13]. Apart from the method of detection by HPLC, none of these techniques employ separation steps capable of resolving or quantitating 7-OH-MTX. In the present paper, we report a relatively simple and sensitive method of detecting MTX and 7-OH-MTX by HPLC in sera and cerebrospinal fluid (CSF) of children receiving high-dose and very-high-dose MTX therapy.

MATERIALS AND METHODS

Chemicals

The chemicals utilized in these studies were obtained from the following sources: MTX and folinic acid in clinical use from Lederle Labs. (Pearl River, NY, U.S.A.); 7-OH-MTX and N-{4-[[(2,4-diamino-6-quinazolinyl)methyl-amino]benzoyl]}aspartic acid were generous gifts from Dr. David Johns and Dr. Harry B. Wood, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.); folic acid, prednisone and prednisolone from Protea (Sydney, Australia); 4-aminoacetophenone from E. Merck (Darmstadt, F.R.G.); trimethoprim from Wellcome Australia (Sydney, Australia); daunorubicin (cerubidin) from May and Baker (Footscray, Australia); purified MTX, cytosine arabinoside and dexamethasone from Sigma (St. Louis, MO, U.S.A.); *p*-aminobenzoic acid from Ajax (Sydney, Australia); HPLC-grade methanol and acetonitrile from Waters Assoc. (Milford, MA, U.S.A.); EMIT methotrexate assay kits from Syva (Palo Alto, CA, U.S.A.). All other chemicals and reagents were of analytical grade.

Chromatographic equipment

HPLC was performed using a Model ALCGPC 204 liquid chromatograph equipped with a U6K injector, a Model 6000A pump and a Model 440

absorbance detector fitted with a 313-nm filter. Chromatography was performed on a Radial-Pak C_{18} column (particle size 10 μm, 10 cm × 5 mm I.D.) fitted into a RCM-100 radial compression separation system. The above pieces of equipment were obtained from Waters Assoc.

Solvent system

The solvent system for column equilibration and drug elution consisted of 0.15 *M* ammonium phosphate buffer (pH 4.85) containing 11% (v/v) acetonitrile. Column elution was performed at room temperature using a flow-rate of 1 ml/min and a pressure of 3500 kPa.

Serum collection

Blood samples were obtained from patients undergoing MTX therapy as a part of the current treatment protocol being utilized in the Section of Paediatric Haematology and Oncology (Prince of Wales Children's Hospital, Sydney, Australia). High-dose methotrexate (HD-MTX), in a dosage of 500—1000 mg/m^2 (one-third of the dose administered in the first 0.5—1 h and the remainder over the next 23 h), was administered intravenously to children with acute lymphoblastic leukemia or lymphoma. Very-high-dose methotrexate (VHD-MTX), in a dosage of 8—12 g/m^2 was administered as an infusion over 4—6 h to children with osteogenic sarcoma. The blood samples were allowed to clot and the serum obtained by centrifugation at 2500 *g* for 15 min.

Sample preparation

Deproteinization of serum samples was performed by passage through a Sep-Pak cartridge (Waters Assoc.,) which is a miniature C_{18} column. This column was previously activated by washing with 10 ml of methanol followed by 10 ml of water. Serum (1 ml) was loaded on to the column and the eluent discarded. Subsequently, the column was washed with 5 ml of water to remove serum proteins and serum contaminants. The free MTX which remained bound to the column was then eluted with 3 ml of methanol. The methanol fraction was dried at 45°C under a jet of nitrogen, and then dissolved in 100 μl of methanol—water (20:80, v/v). This procedure concentrates the MTX present in 1 ml of serum by ten-fold. A 20-μl aliquot of this sample was injected into the chromatograph, and MTX concentrations were determined by measurement of peak heights on HPLC tracings. A standard curve was prepared daily, ranging from $0.11 \cdot 10^{-6}$ *M* to $11 \cdot 10^{-6}$ *M* MTX dissolved in normal drug-free serum. If concentrations in patient sera exceeded the range of the standard curve, they were diluted appropriately. Due to the commercial scarcity of pure 7-OH-MTX, multiple injections of 20 nmol of the pure compound was utilized as a reference standard.

Enzyme multiplied immunoassay technique (EMIT)

Serum MTX concentrations were measured using EMIT [14]. In this procedure, antibodies to MTX, glucose-6-phosphate and the co-enzyme, nicotinamide adenine dinucleotide (NAD), are pipetted into tubes containing sera (50 μl) obtained from patients undergoing either HD-, or VHD-MTX

therapy. Under these conditions, the MTX present in the sera would bind to the added MTX antibodies. In the second step of the assay, the enzyme glucose-6-phosphate dehydrogenase (G6PDH), coupled to MTX, is added to the tubes. The G6PDH—MTX complex binds to any free binding sites still available on the MTX antibodies and thereby becomes inactive in its ability to oxidize glucose-6-phosphate to 6-phosphoglucono-δ-lactone. Therefore a measure of the G6PDH reaction would be in direct proportion to the amount of MTX present in the serum.

$$\text{glucose-6-phosphate} \xrightarrow[\text{NAD}]{\text{G6PDH}} \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADH} + \text{H}^+$$

The G6PDH reaction is determined in a spectrophotometer by measuring the conversion of NAD to NADH, at a wavelength of 340 nm, and at a temperature of 30°C. This method is used to measure MTX concentrations between $3 \cdot 10^{-7}$ and $3 \cdot 10^{-3}$ *M*. Interference from serum G6PDH activity is avoided by use of the co-enzyme NAD, which is active only with the bacterial enzyme (*Leuconostoc mesenteroides*) employed in the assay.

RESULTS

Extraction efficiency of free MTX from serum using the HPLC system

Table I shows that between 20% and 28% of the total MTX was washed off the Sep-Pak during the water wash. Since the binding of added MTX to serum proteins was not measured, we cannot state whether the loss of MTX in the water wash was due to the free MTX, or to protein-bound MTX. Theoretically, the protein-bound drug would not bind to the column and hence would be washed away. Between 67% and 72% of the total MTX was eluted with methanol. The remaining 4—10% was lost, either during serum elution or during the solubilisation procedure. The overall extraction efficiency of free MTX from serum was approx. 70%.

TABLE I

EXTRACTION OF FREE MTX FROM SERUM

Appropriate amounts of MTX (220, 22 or 2.2 nmol) were added to 1 ml of serum and the free MTX was separated and quatitated as described in Materials and methods. The results are expressed as a mean of three experiments.

Amount of MTX (nmol)	220	22	2.2
Amount lost in water wash	62.3	4.3	0.44
Amount eluted with methanol	148.1	16.0	1.54
Miscellaneous loss	9.6	1.7	0.22

Separation of MTX from 7-OH-MTX and other compounds

Fig. 1 shows chromatograms obtained from normal drug-free serum (a); serum spiked with MTX (b); serum spiked with both MTX and 7-OH-MTX (c); serum from a patient 24 h after HD-MTX treatment (d). The present procedure is capable of separating MTX from other contaminants in serum. The retention

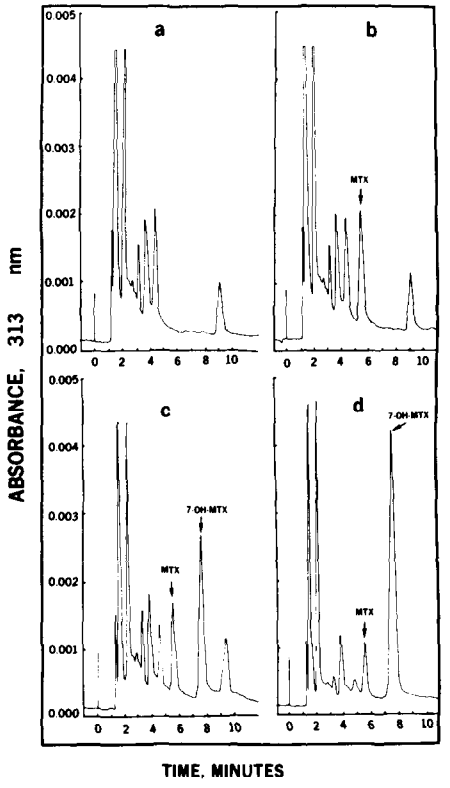

Fig. 1. Representative HPLC chromatograms of normal drug-free serum (a); serum spiked with MTX (b); serum spiked with both MTX and 7-OH-MTX (c); serum from a patient 24 h after HD-MTX treatment (d). Chromatography was performed on a Radial-Pak C_{18} column, as described in Materials and methods.

TABLE II

RETENTION TIMES OF VARIOUS DRUGS AND MTX ANALOGUES

Compound	Retention time (min)
MTX	5.4
7-OH-MTX	7.4
Folic acid	6.6
Folinic acid	2.1
N-{4-[[(2,4-Diamino-6-quinazolinyl)methylamino]-benzoyl]}aspartic acid	4.2
p-Aminoacetophenone	11.8
p-Aminobenzoic acid	3.7
Trimethoprim	16.0
Vincristine	13.6
Daunorubicin	NE*
Cystosine arabinoside	13.2
Dexamethasone	ND**
Prednisone	ND
Prednisolone	ND

*NE = Not eluted 30 min after injection.
**ND = Not detectable at 313 nm.

time of MTX (5.4 min) is sufficiently different from 7-OH-MTX (7.4 min) to be able to accurately quantitate each compound present in serum.

The limit of sensitivity was found to be 4.4 pmol of MTX injected onto the column in a total volume of 20 μl. As the MTX present in 1 ml of serum is concentrated ten-fold, the present method is capable of measuring concentrations of MTX down to $2.2 \cdot 10^{-8}$ *M*. The within-assay coefficient of variation (C.V.) for MTX assay was 1.69%, aand 3.69% for 7-OH-MTX ($n = 6$; 20 nmol). The day-to-day C.V. for MTX was 6.44%, and 7.34% for 7-OH-MTX ($n = 6$; 20 nmol). The C.V. value due to the usage of different Sep-Pak cartridges was investigated. A known concentration of MTX (20 nmol) was injected into a

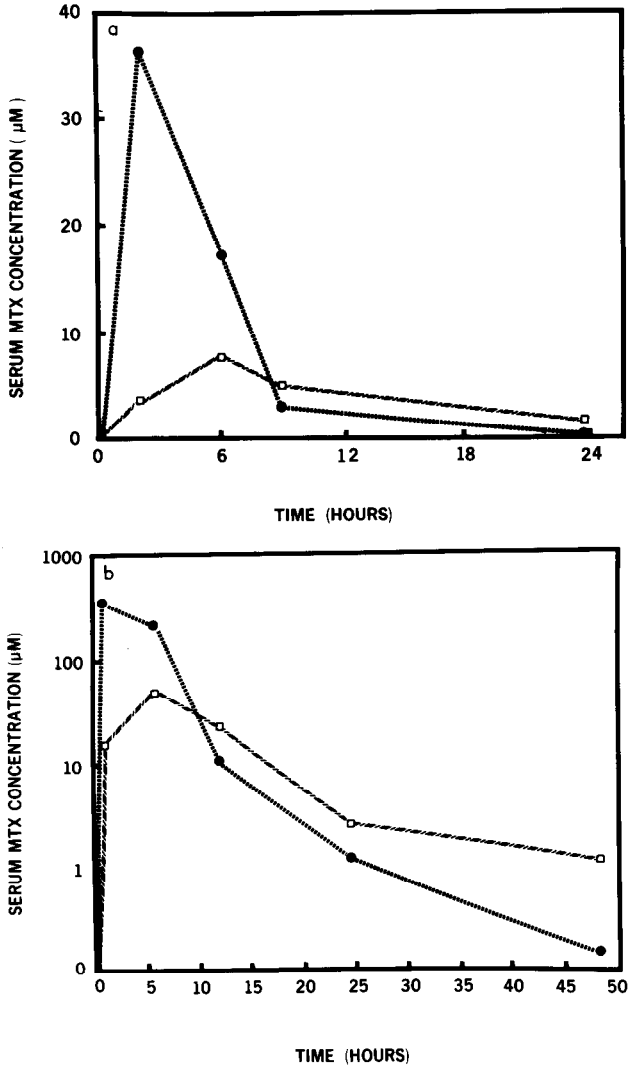

Fig. 2. MTX and 7-OH-MTX concentrations in sera of a patient at selected time intervals following infusion with (a) HD-MTX, 500—1000 mg/m^2; or (b) VHD-MTX, 8—12 g/m^2. The MTX (•) and the 7-OH-MTX (□) concentrations were determined using HPLC as described in Materials and methods.

series of Sep-Pak cartridges and the concentration of eluted drug monitored. The C.V. was found to be 3% (n = 6). This was confirmed by using the radioactive labelled MTX and monitoring the eluted counts.

Table II lists several drugs which may be administered concomitantly with MTX, or compounds with structural similarities to MTX. The steroids do not absorb at 313 nm. Each of the other drugs listed has a distinct retention time to clearly separate it from MTX or 7-OH-MTX.

Concentrations of MTX and 7-OH-MTX in patients

The serum MTX concentration in our patients (Fig. 2 and Table III) were similar to those in other published results [4, 10] and will be the subject of a separate paper [15]. Depending on the actual dosage and body surface area, the peak concentrations achieved in the high-dose regimens ranged from $5 \cdot 10^{-6}$ to $40 \cdot 10^{-6}$ M, and in the very-high-dose regimens these concentrations ranged from $200 \cdot 10^{-6}$ to $600 \cdot 10^{-6}$ M.

TABLE III

SERUM CONCENTRATIONS OF MTX AND 7-OH-MTX IN PATIENTS

MTX and 7-OH-MTX concentrations were measured in the sera of patients at selected time intervals following infusion with HD-MTX, or VHD-MTX.

Patient	Dose*	Time (h)	MTX concentration (μM)	7-OH-MTX concentration (μM)
1	HD	3	8.7	ND**
		6	8.7	ND
		11	6.2	ND
		24	1.1	ND
2	HD	3	37.0	3.5
		6	14.0	7.3
		9	3.2	4.6
		24	0.18	1.0
3	VHD	6	434.0	92.0
		12	16.0	21.0
		24	1.1	2.5
		48	0.19	0.6
4	VHD	6	230.0	106.0
		12	18.0	40.0
		24	1.1	6.5
		48	0.23	2.5
5	VHD	1	550.0	22.0
		6	323.0	70.0
		12	14.0	32.0
		24	1.7	3.7
		48	0.21	1.5

*HD = high-dose treatment, 500—1000 mg/m^2; VHD = Very-high-dose treatment, 8—12 g/m^2.

**ND = not determined.

The administered MTX is metabolised to 7-OH-MTX in vivo, with peak concentrations appearing 6 h after commencement of infusion (Fig. 2 and Table III). The peak concentrations of 7-OH-MTX observed in patients receiving VHD-MTX therapy is high (approx. $70 \cdot 10^{-6}$ *M*). This is a direct consequence of the massive doses of MTX utilized. The effect of such concentrations of 7-OH-MTX on the entry of MTX into the cell, and its effect on the cell remain to be clarified. However, the present experiments do indicate that the excretion rate of 7-OH-MTX is slower than MTX (Fig. 2). This observation would be particularly important with regard to the known low solubility of the drug [2].

The CSF concentrations of MTX 3 h after commencement of high-dose infusion are in the order of 10^{-6} to 10^{-7} *M*. In exceptional cases CSF concentrations of $3 \cdot 10^{-6}$ to $4 \cdot 10^{-6}$ *M* have been found.

Comparison between HPLC and EMIT for determination of MTX concentrations in serum

Serum MTX concentrations were determined concurrently using EMIT and HPLC. Linear regression analysis of the data in Fig. 3 shows a very high degree of correlation ($r = 0.97$, $p < 0.001$) between the two procedures. The gradient of the graph being close to unity ($m = 0.99$) suggests that the two methods are furnishing similar values for the MTX concentrations.

The cross-reactivity of 7-OH-MTX binding site on the MTX antibody was investigated using EMIT. A known concentration of 7-OH-MTX ($2.2 \cdot 10^{-6}$ *M*) was incubated with the MTX antibody. The MTX concentration determined

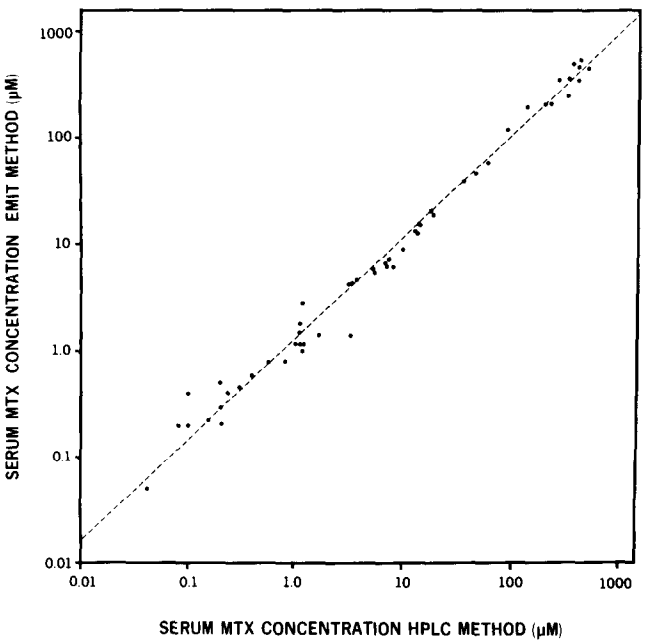

Fig. 3. Comparison between HPLC and EMIT for determination of MTX concentrations in serum. MTX concentrations were determined by HPLC and EMIT in sera obtained from patients at selected time intervals following either HD-, or VHD-MTX infusion ($r = 0.97$, $p < 0.001$).

was $0.08 \cdot 10^{-6}$ *M*, i.e. a cross-reactivity of 4%. This shows that 7-OH-MTX present in sera does not bind to the MTX antibody, and therefore does not influence the levels of MTX determined in the sera by EMIT.

DISCUSSION

Measurement of MTX concentrations in serum by methods such as enzyme inhibition [3, 4], protein binding [5], RIA [6, 7] and fluorescence [8, 9] are useful for clinical investigations, but are of limited value for metabolic studies. Use of HPLC allows for the measurement of metabolites such as 7-OH-MTX [10—13]. In patients receiving HD-MTX therapy, 7-OH-MTX is present in significant quantities. However, the biochemical and clinical importance of this compound still needs to be clarified.

With HPLC, serum samples have to be deproteinized prior to injection onto a column. When methods such as organic extraction [10], ammonium sulphate precipitation [13], and acid precipitation [11, 12] are used, deproteinization can be laborious and time-consuming. The utilization of Sep-Pak C_{18} cartridges for deproteinization allows for the removal of serum proteins and other polar metabolites in a single step. The MTX and 7-OH-MTX which are retained on the column can be concentrated from 2—5 ml of serum, allowing low levels of the drug and its metabolites to be measured.

In our method, the overall efficiency of free MTX extraction from serum is 70% (Table I). This compares favourably with other reported values which range from 46% to 82% [10, 11, 13]. The limit of sensitivity is $2.2 \cdot 10^{-8}$ *M*. This is similar to the value of $1 \cdot 10^{-8}$ to $2 \cdot 10^{-8}$ *M* reported by Canfell and Sadée [10], and is ten-fold better than other reports [11, 13]. The increased sensitivity can be attributed to the ten-fold concentrating step achieved by using Sep-Pak, the use of acetonitrile rather than methanol in the elution buffer, and the increased absorbance of MTX at 313 nm in comparison to 254 nm (the absorbance maximum of methotrexate is 305 nm).

We have measured MTX concentrations in serum using RIA [16], EMIT and HPLC, using both the radial compression module (RCM) and stainless-steel columns. They all furnish similar results and each method has particular advantages and disadvantages. The EMIT and RIA methods appear to be suited to clinical laboratories dealing with large numbers of samples. The HPLC method appears to be better suited to research laboratories. We found the RCM module particularly attractive as compared with stainless-steel columns; the cartridges are cheaper, easier to attach, and easier to store without any apparent loss in efficiency. A particular disadvantage of the RIA and EMIT methods is that the kits are quite expensive and have expiry dates for radioisotopes, enzymes and antibodies. This can result in significant waste when only small numbers of samples are being processed.

We believe HPLC provides a method for measuring MTX and its metabolites, which is technically straightforward, economical, but with a high degree of specificity and sensitivity. In addition the present method saves time, requiring less than 15 min to process each sample.

ACKNOWLEDGEMENTS

We thank the children at the Prince of Wales Children's Hospital for co-operating in this study, and the medical and the nursing staff for providing the blood specimens. We also thank the staff of the Department of Medical Illustration, University of N.S.W. and Teaching Hospitals for their assistance. This study was supported by a grant from the Children's Leukaemia and Cancer Research Foundation.

REFERENCES

1 S. Salasoo, M.G. Irving, R. Lam-Po-Tang, D. O'Gorman Hughes and A. Freedman, Med. J. Aust., 1 (1976) 777.
2 S.A. Jacobs, R.G. Stoller, B.A. Chabner and D.G. Johns, J. Clin. Invest., 57 (1976) 534.
3 W.C. Werkheiser, S.F. Zakrzewski and C.A. Nichol, J. Pharm. Exp. Ther., 137 (1962) 162.
4 Y.M. Wang, E. Lantin and W.W. Sutow, Clin. Chem., 22 (1976) 1053.
5 C.E. Myers, M.E. Lippman, H.M. Eliot and B.A. Chabner, Proc. Nat. Acad. Sci. U.S., 72 (1975) 3683.
6 V. Raso and R. Schreiber, Cancer Res., 35 (1975) 1407.
7 C. Bohuon, F. Duprey and C. Boudene, Clin. Chim. Acta, 57 (1974) 263.
8 S.G. Chakrabarti and I.A. Bernstein, Clin. Chem., 15 (1969) 1157.
9 J.M. Kincade, W.R. Volger and P.G. Dayton, Biochem. Med., 10 (1980) 337.
10 C. Canfell and W. Sadée, Cancer Treat. Rep., 64 (1980) 165.
11 G.J. Lawson, P.F. Dixon and G.W. Aherne, J. Chromatogr., 223 (1981) 225.
12 J. Jolivet and R.L. Schilsky, Biochem. Pharm., 30 (1981) 1387.
13 J.L. Cohen, G.H. Hisayasu, A.R. Barrientos, M.S. Balachandran Nayar and K.K. Chan, J. Chromatogr., 181 (1980) 478.
14 Methotrexate Assay EMIT® and TM, Instruction Manual, Syva, Palo Alto, CA.
15 N. So, D.P. Chandra and D.W. O'Gorman-Hughes, in preparation.
16 Methotrexate (^{125}I) Radioimmunoassay Kit, Instruction Manual, Diagnostic Biochemistry, San Diego, CA.