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COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THE ABBOTT FLUORESCENT POLARIZATION RADIOIMMUNOASSAY IN THE MEASUREMENT OF METHOTREXATE

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SUMMARY

A modified high-performance liquid chromatographic (HPLC) technique for the assay of methotrexate is described and compared to the Abbott Fluorescence Polarization Radioimmunoassay The reproducibility (coefficient of variation) at low concentrations was similar for the two assays 8 1 and 8 5% for the Abbott and HPLC assay, respectively The limit of detection of the two assays was also similar at 0 01 μM The correlation coefficient for Abbott versus HPLC was 0 9833 with a gradient of 0 9545 Aspirin was the only drug that interfered with HPLC Methotrexate's metabolite 7-hydroxymethotrexate did not interfere with the Abbott assay Plasma half-lives were similar to oncology patients in the two rheumatological patients studied The 7-hydroxymethotrexate half-life was 15 h

INTRODUCTION

Methotrexate (MTX), an anti-metabolite cytotoxic, has been a useful agent in the treatment of rheumatological and oncological disorders In order to improve the response of tumours to methotrexate high-dose infusions have been used [1], unfortunately this is commonly associated with marrow and gastrointestinal toxicity Folinic acid which bypasses the dihydrofolate reductase inhibition induced by methotrexate is administered 24 h later as an antidote to high-dose infusions Plasma methotrexate levels greater than 10^{-6} and 10^{-7}

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M, measured at 24 and 48 h, respectively, have been found to correlate with toxicity, and the duration of folinic acid rescue can be modified accordingly [2–4]

Methotrexate is metabolized in the liver to a number of derivatives; it is primarily hydroxylated to 7-hydroxymethotrexate (7-OH-MTX) and secondly to a number of polyglutamyl derivatives Both 7-OH-MTX and the polyglutamyl derivatives have been found to be active. The ability of 7-OH-MTX to inhibit dihydrofolate reductase is approximately a quarter that of methotrexate at equimolar concentrations [5,6] and in patients with acute leukaemia the infusion of high-dose methotrexate has resulted in levels of 7-OH-MTX that have exceeded those of the parent compound [6–8] Therefore in measuring methotrexate levels the 7-hydroxy derivative must be separated from the parent compound if clinical toxicity is to be correlated with plasma levels.

Many assays have been devised to measure methotrexate and include radioimmunoassays [9], high-performance liquid chromatography (HPLC) [3,5] and fluorescence techniques [10]. This study compared our modification of Colliers HPLC assay [11] and the Abbott Fluorescence Polarization Radioimmunoassay to determine whether 7-OH-MTX interfered with the Abbott kit measurement of methotrexate Also, preliminary pharmacokinetic data are presented in patients with rheumatological conditions receiving low-dose methotrexate and compared to those obtained in oncological patients. Since the co-administration of aspirin with methotrexate is known to prolong the elimination half-life of methotrexate this study presents preliminary data on the combination of indomethacin with methotrexate

EXPERIMENTAL

Instrumentation

HPLC was performed on a Waters liquid chromatography pump, (Waters, Richmond, Australia), a Waters reversed-phase Radial Pack (RCM-100) C_{18} column and a Waters 450 variable-wavelength detector A Sep-Pak C_{18} precolumn was used for methotrexate extractions The Abbott Fluorescence Polarization Radioimmunoassay was donated by Abbott (Kew, Australia)

Materials and reagents

8-Chlorotheophylline (8-CT) was purchased from Sigma (St Louis, MO, U S.A) Methotrexate was obtained from Lederle and 7-OH-MTX was a gift from Dr G Johns of Bethesda Hospital (Bethesda, MD, U S A)

Patient selection and blood collection

Six patients suffering from the following rheumatological conditions (polymyositis, rheumatoid arthritis and psoriatic arthritis) treated with low-dose oral methotrexate were studied The results from two patients receiving meth-

otrexate and indomethacin in combination are also presented These two patients received intravenous methotrexate (20-mg bolus) on two occasions firstly while on no other medication and secondly while taking indomethacin All patients had a full blood examination and renal assessment including 24-h urine collection for creatinine clearance prior to investigation A 10-ml volume of blood was collected in EDTA tubes at 0, 2, 5, 15, 30, 60 and 90 min and 2, 3, 4, 6, 8 and 24 h The blood was centrifuged and the plasma stored at $-4°C$ The plasma samples were processed on the same day by both the Abbott kit and HPLC to exclude alterations in methotrexate levels occurring due to repeated thawing

Extraction procedure

To 1 920 ml of plasma were added 80 μl of internal standard (8-CT, 1 mg/ml) and 200 μl of 35% perchloric acid The mixture was vortex-mixed and centrifuged at 2000 *g* for 20 min, and the supernatant was recovered and applied to the prepared Sep-Pak C_{18} pre-column as described below

After the Sep-Pak columns were prewashed with 10 ml of methanol, 10 ml of water and 10 ml of 0 02 *M* acetate buffer, pH 5, the supernatant was diluted in 10 ml 0 02 *M* acetate buffer and applied to the Sep-Pak pre-column The pre-column was again washed with 10 ml of water and 2 ml of 10% methanol in water Methotrexate was eluted off the column with 100% methanol and dried at 60°C under nitrogen The residue was redissolved in 100 μl of 5 m*M* hydrochloric acid and applied to the HPLC system This extraction procedure resulted in a twenty-fold increase in the methotrexate concentration in the final solution.

Chromatography conditions

The mobile phase was acetonitrile–10 m*M* potassium dihydrogenphosphate (12 5 87 5) at pH 3 9 The pump was run at 2 5 ml/min and the detector set at 313 nm. This wavelength, although not the most sensitive, resulted in a significant reduction of the early plasma peaks, thereby minimizing the interference with the internal standard However, no significant reduction in sensitivity, i e greater than 5%, occurred

Abbott assay

The Abbott assay is a totally automated system and requires simple preparation of the plasma samples prior to analysis by the Abbott TDX unit

RESULTS

The chromatogram of blank plasma demonstrates the solvent front peak and two early plasma peaks (see Fig 1) Fig 2 illustrates the resulting chro-

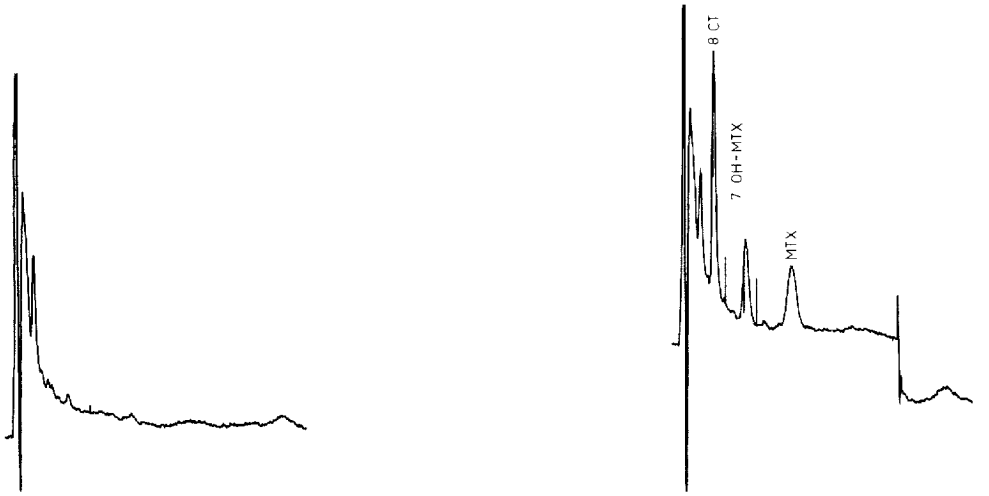

Fig 1 Chromatogram from extracted drug-free plasma

Fig 2 Chromatogram representing plasma spiked with internal standard (8-CT), 7-OH-MTX (42 ng/ml) and MTX (31 ng/ml) The three compounds have all eluted by 8 min There is an occasional late plasma peak not related to the above compounds which occurs at 14 min

TABLE I

LIMIT OF DETECTION, REPRODUCIBILITY, RECOVERY AND PLASMA VOLUME REQUIRED FOR THE TWO ASSAYS

The two assays have similar limits of detection and reproducibility at the lower concentrations

Assay	Limit of detection (μM)	Reproducibility (%)	C V (%)	Recovery (%)	Plasma volume
Abbott	0 01	8 1 (at 0 07 μM)	0 96 (at 0 8 μM)	98	100 μl
HPLC	0 015	MTX 8 5 (at 0 04 μM)	8 5 (at 0 9 μM)	40–48	2–3 ml
		7-OH-MTX 7 1 (at 0 04 μM)	8 5 (at 0 8 μM)		

matogram following spiking of plasma with internal standard (8-CT), 7-OH-MTX and methotrexate, the retention times being 2 7, 5 and 8 min, respectively

Limit of detection

Following the extraction procedure and a resultant twenty-fold increase in the final concentration of the sample the limit of detection for methotrexate and 7-OH-MTX using HPLC was 0 015 μM This compares favourably with the Abbott detection limit of 0 01 μM (see Table I) A pharmacokinetic profile of a patient is included using the Abbott and the HPLC assays (Fig 3)

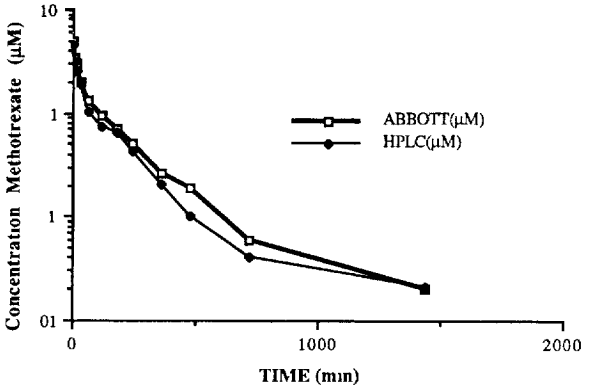

Fig 3 Methotrexate pharmacokinetics in a patient using both Abbott and HPLC assays

Reproducibility

The reproducibility on HPLC as determined by the coefficient of variation (standard deviation/mean) was performed on twenty samples, ten at 0 04 μM and ten at 0 8 μM The correlation of variation for methotrexate and 7-OH-MTX at 0 04 μM was 7 1% compared to 8 1% for the Abbott kit

The coefficients of variation (C V) at 0 8 μM for the Abbott kit and HPLC were 0 96 and 8 5%, respectively (see Table I)

Recovery

The recovery was calculated by the following formula

$$\text{Recovery} = A/B \times K \times 100 \times C/D$$

where A = peak height of extracted compound at concentration X, B = peak height of unextracted compound at concentration Y, C = concentration of the extracted compound (X), D = concentration of the unextracted compound (Y) and K = concentration factor (20)

The recovery for HPLC was 46% compared to 96% for the Abbott kit

Comparison of plasma levels

The comparison of the two assays performed on a total of 45 samples demonstrated a correlation coefficient of 0 9833 and a slope of 0 9545 (Fig 4) Plasma methotrexate levels determined by both methods were very similar and plasma spiked with 7-OH-MTX did not interfere with the methotrexate level obtained using the Abbott kit

Interfering drugs

The following drugs did not interfere with the HPLC method naproxen, indomethacin, triamterene, dihydrallazine, leucovorin, cyclophosphamide,

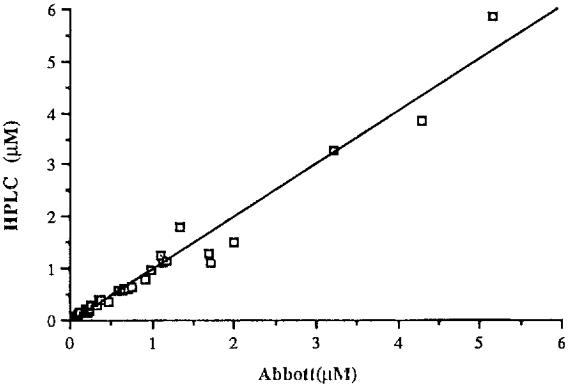

Fig 4 Methotrexate concentrations of 45 samples in 3 patients with rheumatological conditions The results of the HPLC assay (y-axis) were plotted against those of the Abbott kit (x-axis) The coefficient of correlation and gradient were 0 9833 and 0 9545, respectively

adriamycin, vincristine and 1-asparaginase Aspirin, however, produced a large peak which interfered with the internal standard This peak occurred at pharmacological levels of aspirin

Adriamycin and cyclophosphamide did not interfere with the Abbott kit

Pharmacokinetic data

In all patients receiving oral or intravenous methotrexate, results of full blood investigations and renal function were normal The β half-lives for the six patients receiving methotrexate orally were 4 5 ± 1 2 h (mean ± S E M) for

TABLE II

RESULTS OF α AND ELIMINATION HALF-LIVES AND AREAS UNDER THE CURVE FOR METHOTREXATE AND 7-HYDROXYMETHOTREXATE

The AUC of methotrexate did not change with or without indomethacin administration, however, there was a large difference in patient's conversion of methotrexate to 7-hydroxymethotrexate

	Half-life MTX (h)	Elimination half-life (h)		AUC (μM min)		Ratio AUC 7-OH-MTX/MTX
		MTX	7-OH-MTX	MTX	7-OH-MTX	
Patient 1 (MTX alone)	0 76	3 96	15 12	493	152	0 31
Patient 1 (MTX and indomethacin)	0 96	5 18	14 33	331	196	0 59
Patient 2 (MTX alone)	0 66	4 19	16 60	374	305	0 82
Patient 2 (MTX and indomethacin)	0 93	4 92	15 59	402	757	1 88

MTX and 14.55 ± 1 h for 7-OH-MTX. The results of the α and β half-lives and the areas under the curve (AUC) for methotrexate and 7-OH-MTX for the two patients receiving methotrexate and indomethacin are presented in Table II. The mean ± S E M of the α half-lives for methotrexate was 0 83 ± 0.07 h The mean ± S E.M of the elimination half-lives for methotrexate was 4 60 ± 0 30 h and that of 7-OH-MTX was 15.4 ± 0.47 h The hydroxy metabolite of methotrexate would appear 45–60 min after methotrexate whether administered intravenously or orally The AUC for methotrexate was 400 ± 34 μM min and for 7-OH-MTX was 369 ± 137 μM min The ratios of the AUC are given in Table II. The oral bioavailability performed in three other patients was 59 ± 3% which is comparable to that found in oncological patients.

CONCLUSION

Surprisingly the Abbott kit was specific for methotrexate and was not affected by the presence of 7-OH-MTX in the samples The limits of detection in the two assays were similar, furthermore, the Abbott kit had an extremely good correlation of variation at higher concentrations compared to HPLC The Abbott kit was easier to perform, required only small volumes of plasma for assay, afforded the same level of detection and correlated well with the HPLC method but was unable to measure 7-OH-MTX and is more expensive

Unfortunately, aspirin gave an early peak which interfered with the internal standard in the HPLC assay, however, other anti-inflammatory and cytotoxic drugs did not interfere with the measurement of methotrexate and 7-OH-MTX in the HPLC assay

The pharmacokinetics of methotrexate in these initial two rheumatological patients demonstrated a short α half-life phase of 0 83 h with an elimination half-life of 4 6 h and an even longer 7-OH-MTX elimination half-life of 15 h which compares with that of oncological patients [3] The elimination half-life of methotrexate of 4.6 h is still short compared to some other reports [3,5], this may reflect the use of high-dose infusions in oncological patients or that 7-OH-MTX may have contributed to the half-life

The AUC for methotrexate and 7-OH-MTX in these two patients are presented in Table II and there is considerable inter-individual variation These findings have been previously reported in leukaemic patients and patients treated with methotrexate for solid tumours where the methotrexate/7-OH-MTX AUC ratio varied between 0 5 and 1 59 [6]

The increase in AUC for patient 2 while taking the combination of methotrexate and indomethacin was not associated with an increase in the elimination half-life and therefore cannot be correlated with decreased renal excretion The actual levels of 7-OH-MTX were significantly higher in this patient implying an increased hepatic conversion of MTX

The role of drugs such as alcohol or barbiturates that will induce hepatic

enzymes, or even methotrexate itself, may be important in the conversion of methotrexate to 7-OH-MTX Since there is such an inter-individual variability in AUC for 7-OH-MTX and because of its long elimination half-life, the possibility that methotrexate toxicity may be partly related to this metabolite cannot be totally disregarded This may help explain the variability in toxicity seen when plasma methotrexate levels are non-toxic but patients manifest toxicity

We are currently performing further investigations with methotrexate toxicity

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