Journal of Chromatography, 488 (1989) 379–388
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4572

STABLE AND SENSITIVE METHOD FOR THE SIMULTANEOUS DETERMINATION OF N^5-METHYLTETRAHYDROFOLATE, LEUCOVORIN, METHOTREXATE AND 7-HYDROXYMETHOTREXATE IN BIOLOGICAL FLUIDS

O. VAN TELLINGEN*, H.R. VAN DER WOUDE, J.H. BEIJNEN, C.J.T. VAN BEERS and W.J. NOOYEN

Department of Clinical Chemistry, Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam (The Netherlands)

(Received October 4th, 1988)

SUMMARY

A high-performance liquid chromatographic method for the determination of N^5-methyltetrahydrofolic acid, leucovorin, methotrexate and 7-hydroxymethotrexate in plasma and liquor samples is presented. Gradient elution is used to increase the sensitivity. Four sample preparation methods were compared with respect to the stability of the injectable sample. Samples can be pretreated with a simple deproteinization method. For enhanced selectivity a solid-phase extraction procedure is described.

INTRODUCTION

Methotrexate (MTX) is widely used in the treatment of several human cancers. The drug acts as an inhibitor of dihydrofolate reductase (DHFR) (EC 1.5.1.3), thereby decreasing the intracellular pool of tetrahydrofolic acid (THFA) analogues and exerting cytotoxicity. The introduction of high-dose MTX therapy, with leucovorin [citrovorum factor (CF)] rescue, has improved the therapeutic efficacy of this agent. Drug-related toxicity can be predicted by monitoring plasma levels and prevented by administration of supplemental CF [1]. The major metabolic product found in plasma is 7-hydroxymethotrexate (7-OH-MTX).

Previous methods reported for the determination of MTX in biological matrices are numerous and include radioimmunoassays (RIA) [2], enzymatic assays with DHFR [3] and high-performance liquid chromatographic (HPLC) assays [4]. Eksborg and Ehrsson [5] reviewed the literature on the bioanalysis of MTX. Few papers have dealt with the determination of CF and its major metabolic

0378-4347/89/$03.50

product N^5-methyltetrahydrofolic acid (N^5-methyl-THFA) in biological matrices. These compounds are reported to be unstable owing to oxidation, and addition of ascorbic acid to the samples is generally recommended [6–8]. However, none of the papers cited provided data concerning the stability of these compounds during the analysis. CF has been determined using RIA [2] and HPLC [6,8]. HPLC provides the best accuracy and possesses sufficient sensitivity combined with speed. Further, HPLC has the potency for the simultaneous determination of CF and MTX and their metabolites.

This paper describes a sensitive HPLC method suitable for the simultaneous determination of MTX, 7-OH-MTX, CF and N^5-methyl-THFA. Several sample preparation methods have been tested. Special attention was paid to optimizing the assay with respect to stability.

EXPERIMENTAL

Instrumentation and chromatography

Chromatographic analyses were performed using an HPLC system consisting of two Spectroflow 400 pumps, a Spectroflow 757 variable-wavelength UV detector operating at 305 nm (Kratos, NJ, U.S.A.), a Model 5140 solvent programmer (Kipp & Zonen, Delft, The Netherlands) and a Model CR-3A integrator (Shimadzu, Kyoto, Japan). Either an MSI 660 automatic sampling device (Kontron, Zurich, Switzerland) with a 100-μl loop or a Rheodyne 7125 injector provided with a 1-ml loop was used.

Samples were chromatographed on a glass column (20 cm $\times$ 3.0 mm I.D.) packed with 5-μm Hypersil-ODS (Chrompack, Middelburg, The Netherlands). All buffers used for chromatography were filtered through 0.45-μm cellulose acetate (HAWP) (Waters Assoc., Milford, MA, U.S.A.). Immediately before use all mixtures were degassed under vacuum. Solvent A consisted of 10 m*M* ammonium formate buffer and solvent B was 25% (v/v) acetonitrile in solvent A, both at pH 3.5 adjusted with hydrochloric acid. The flow-rate of the mobile phase was 0.4 ml/min. The samples were eluted against a linear gradient from 15% to 95% mobile phase B in 21 min, followed by 1 min with 95% mobile phase B. Subsequently the column was re-equilibrated with 15% mobile phase B. The total analysis time was 33 min.

Solid-phase extractions were performed on SPE C_{18} (1 ml) columns (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns were preconditioned according to the manufacturer's instructions.

Solvents and reagents

MTX, N^5-methyl-THFA, aminopterin (AMT) and ammonium formate were purchased from Sigma (St. Louis, MO, U.S.A.) and leucovorin from Duchefa (Haarlem, The Netherlands). Only a small amount of a solution of 7-OH-MTX (24.3 μg/ml) was available, which was a gift from Repgo TNO (Rijswijk, The Netherlands). All other solvents and reagents were obtained from E. Merck (Darmstadt, F.R.G.) and were of analytical-reagent grade (except acetonitrile, LiChrosolv quality). Water was purified with a Millipore-Q system (Waters Assoc.).

Handling of blood, plasma and cerebrospinal fluid samples

Blood samples were collected in glass tubes containing heparin or EDTA as anticoagulant and plasma was obtained by centrifugation (2000 *g*) immediately after sampling. Ascorbic acid (1 mg/ml) was added to the samples, which were stored at −20°C until analysis. Cerebrospinal fluid was also kept at −20°C until analysis.

Sample preparation methods

Method A [7]. To 0.9 ml of plasma, 0.1 ml of ascorbic acid solution (10 g/l) was added and the mixture was allowed to equilibrate for 3 min. With vigorous mixing, 1 ml of trichloroacetic acid (TCA) solution (100 g/l in 0.1 *M* hydrochloric acid) was added. After centrifugation at 2000 *g* for 5 min, 100 μl of the clear supernatant fluid were injected into the chromatographic system.

Method B [6]. A 190-μl volume of plasma (containing 2 mg/ml ascorbic acid and 0.2 *M* 2-mercaptoethanol) was mixed with 10 μl of 500 g/l TCA solution in water and centrifuged for 10 min (15 000 *g*, 4°C). The supernatant fluid (100 μl) was injected directly on to the column.

Method C (perchloric acid method; this work). To 250 μl of plasma (or cerebrospinal fluid) 25 μl of 10 g/l ascorbic acid solution were added and the mixture was vortex-mixed with 250 μl of ice-cold 1.5 *M* perchloric acid, then placed in ice–water for 5 min. After centrifugation for 5 min (3000 *g*, 4°C), 350 μl of the supernatant solution were mixed with 50 μl of 8 *M* potassium acetate solution. This mixture was kept on ice–water for 2 min, then centrifuged for 2 min (3000 *g*, 4°C) and 100 μl of the supernatant fluid were injected.

Method D (solid-phase extraction method; this work). A 500-μl volume of plasma was mixed with 50 μl of 10 g/l ascorbic acid solution, 10 μl of 100 μ*M* AMT solution (internal standard) and 500 μl of 5% (v/v) acetic acid. This mixture was placed on a preconditioned SPE C_{18} (1 ml) column, which was washed with 2 ml of 10 m*M* ammonium formate buffer (pH 3.5) containing 1 g/l ascorbic acid. The column was not allowed to dry. Elution was performed with a mixture of 5% (v/v) acetonitrile and 1 g/l ascorbic acid in 10 m*M* phosphate buffer (pH 6). Other proportions of acetonitrile and buffer were tested (see Results). A 100-μl volume of the eluate was injected. Up to 1 ml can be injected on to the analytical column after adjusting the pH of the eluate to 3.5 with 0.1 *M* hydrochloric acid.

Calibration

Calibration was performed by assaying blank plasma samples spiked with N^5-methyl-THFA, CF and MTX. These standards also contained 1 g/l ascorbic acid. The standard used for MTX analysis in cerebrospinal fluid was a solution of MTX in water. For the determination of 7-OH-MTX an aqueous solution of this compound was used.

RESULTS

Several sample preparation methods were tested. Fig. 1 shows the chromatograms obtained after sample pretreatment according to method A. The peak that

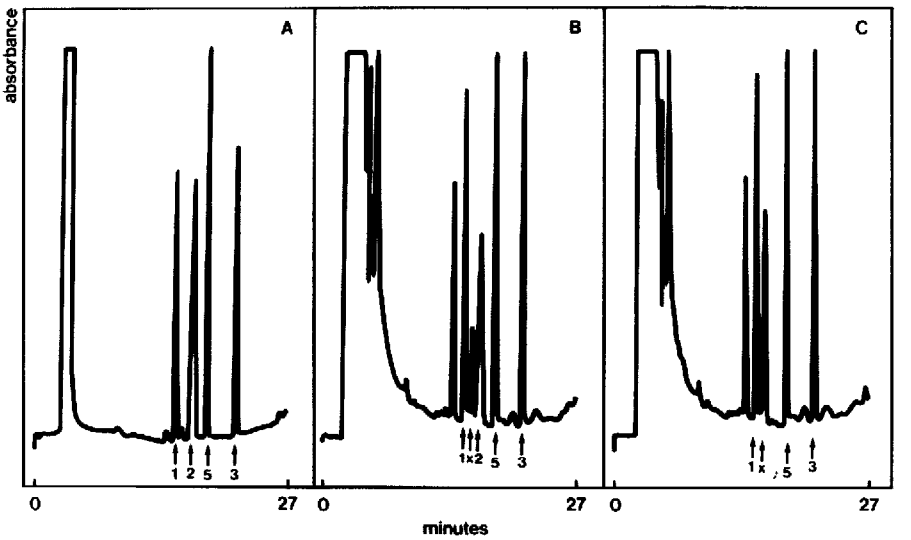

Fig. 1. Conversion of leucovorin in samples deproteinized with TCA. Chromatogram A represents a mixture of (1) N^5-methyl-THFA, (2) CF, (3) MTX and (5) AMT in water. A plasma sample was spiked with the four compounds, subjected to method A (deproteinized with TCA) and analysed immediately (chromatogram B) and 1 h later (chromatogram C). The broad peak of CF (2) disappears on standing and a sharp peak with a shorter retention time (X) appears.

corresponds to CF disappeared within 1 h and a new, much sharper peak that has a slightly shorter retention time appeared. We spiked samples with increasing concentrations of CF. When these samples were pretreated according to method A, left for 1 h at room temperature and then chromatographed, a linear relationship ($r=0.999$) was found between the amount of CF spiked and the area of this new peak. The same conversion of CF occurred when sample preparation method B was used.

Addition of ascorbic acid prior to deproteinization with perchloric acid (method C) is necessary, otherwise the recoveries of N^5-methyl-THFA and CF are low and variable. Prolonged incubation with perchloric acid further decreases the recovery of CF (Table I).

Plasma samples were deproteinized using solid-phase C_{18} extraction (SPE) columns (method D). When plasma samples were applied directly on to a SPE column, about 5–10% of all the components of interest were rinsed off; however, previous dilution of the sample (1:1) with 5% (v/v) acetic acid made their retention complete (verified by HPLC). The retention of the various components on SPE C_{18} columns was limited at pH 5–7 and this property was used to obtain selective elution. Fig. 2 shows the different elution profiles for CF, N^5-methyl-THFA, MTX and AMT using various mixtures of acetonitrile and phosphate buffer at pH 6. For the quantitative recovery of MTX, 3 ml of acetonitrile–buffer (7.5:92.5) is needed, whereas only 2 ml of acetonitrile–buffer (5:95) are sufficient for CF. The recoveries of N^5-methyl-THFA and AMT are then approximately 90% and 85%, respectively.

TABLE I

RECOVERIES OF N^5-METHYL-THFA, CF AND MTX

Plasma samples were deproteinized using method C. The concentration of the ascorbic acid solution added to the sample (25 μl per 250 μl of plasma) was varied from 0 to 50 g/l and the incubation times in ice-water were 5, 10 and 20 min. The recoveries are given as percentages relative to a standard solution in water.

Compound	Incubation time (min)	Concentration of ascorbic acid (g/l)				
		0	5	10	20	50
N^5-Methyl-THFA	5	18	91	89	94	91
	10	19	91	92	97	94
	20	20	87	90	94	91
CF	5	35	60	62	65	66
	10	29	57	57	56	58
	20	29	45	48	51	52
MTX	5	87	89	85	84	80
	10	86	86	83	83	80
	20	86	84	83	82	81
7-OH-MTX	5	—	—	77	—	—

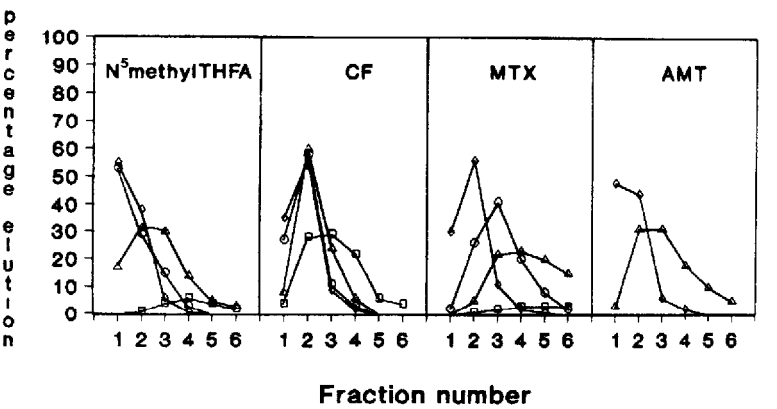

Fig. 2. Elution profiles of N^5-methyl-THFA., CF, AMT and MTX on solid-phase C_{18} extraction columns. Spiked plasma samples [diluted 1:1 with 5% (v/v) acetic acid] were passed through four SPE C_{18} columns, each being rinsed with 2 ml of 10 m*M* ammonium formate buffer (pH 3.5). Each column was eluted with different ratios of acetonitrile to 10 m*M* phosphate buffer (pH of the mixtures = 6) (□ = 2.5:97.5; △ = 5:95; ○ = 7.5:92.5; ◇ = 10:90). For each compound the elution profiles were established by analysing six fractions of 0.5 ml of eluate.

The stability of samples obtained after pretreatment according to methods C and D is shown in Fig. 3. The stability of the samples pretreated according to method C is better than that of samples pretreated by using method D. Storage of samples at 4 °C prior to analysis or the addition of large amounts of ascorbic acid subsequent to the pretreatment procedure (0.1 ml of 100 g/l ascorbic acid

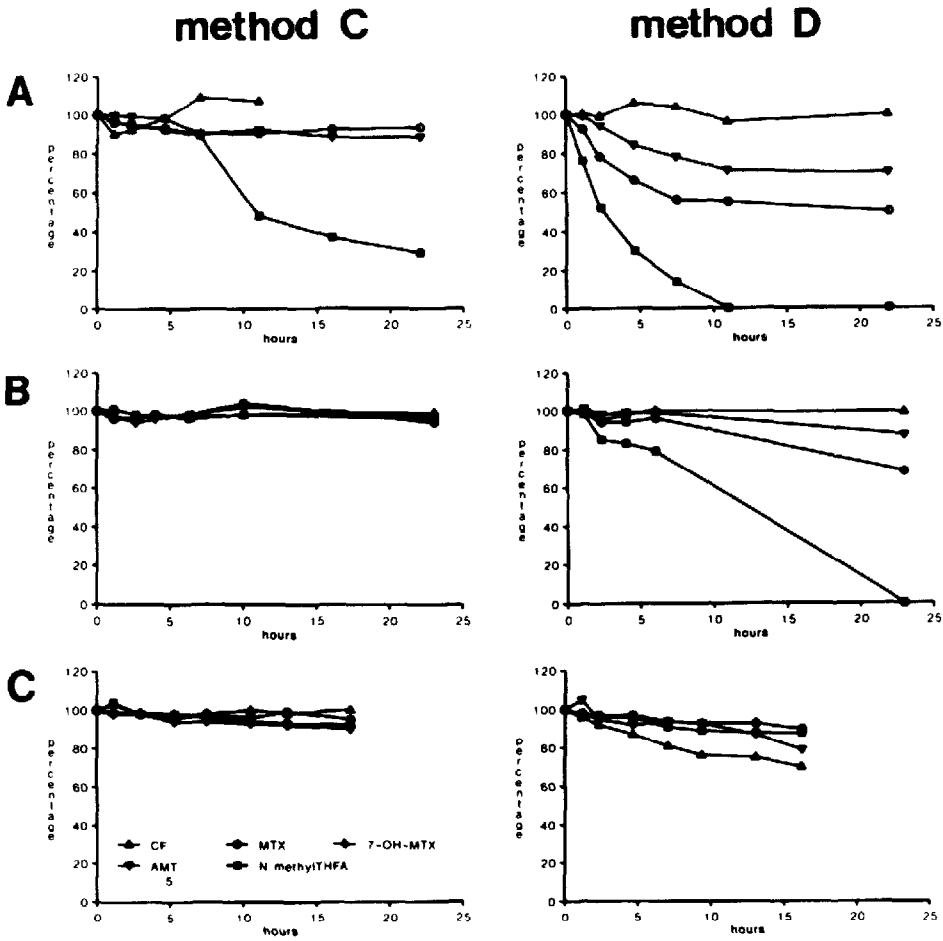

Fig. 3. Long-term stability of N^5-methyl-THFA, CF, MTX and AMT in pretreated samples. Spiked samples were pretreated according to methods C and D The stability of the compounds was established keeping the samples for injection under various conditions: (A) no further treatment (samples left at room temperature), (B) kept at 4 °C until analysis or (C) addition of 0.2 ml of 100 g/l ascorbic acid to 2.0 ml and kept at room temperature. Aliquots were analysed with HPLC at various times. The concentrations in the subsequent aliquots are given relative to the concentration found immediately following sample pretreatment (0 h). The stability of 7-OH-MTX was determined only in samples pretreated with method C and kept under conditions C.

per millilitre of pretreated sample) yields relatively stable solutions suitable for HPLC analysis.

The detection limits are $0.8 \cdot 10^{-12}$ mol for N^5-methyl-THFA, $0.5 \cdot 10^{-12}$ mol for CF and $0.7 \cdot 10^{-12}$ mol for MTX and 7-OH-MTX injected on-column (signal-to-noise ratio = 5:1).

Typical chromatograms of blank and spiked samples obtained after sample preparation methods C and D are shown in Fig. 4. These chromatograms show that detection at 305 nm is superior to that at 285 nm in terms of selectivity.

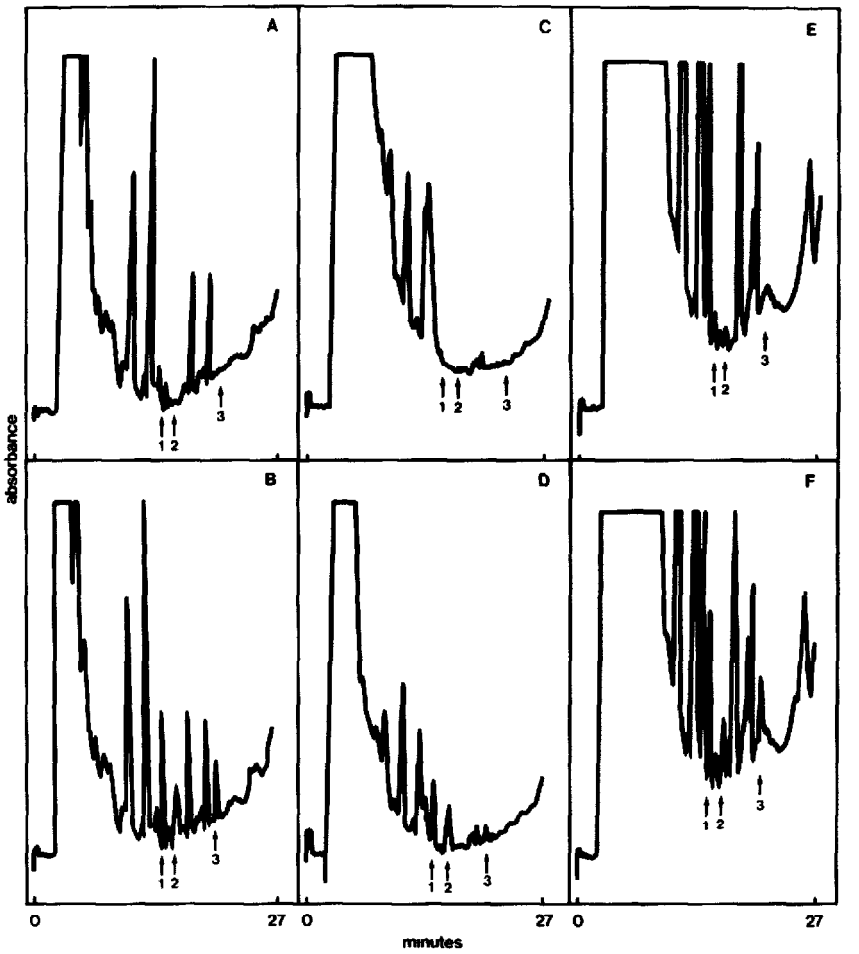

Fig. 4. Detection limits in plasma samples. A blank sample (A, C, E) and the same sample spiked with N^5-methyl-THFA, CF (200 n*M*) and MTX (50 n*M*) (B, D, F) were pretreated according to methods C (A, B) and D (C, D). The first groups were rechromatographed with detection at 285 nm (instead of 305 nm). Although the responses for CF and N^5-methyl-THFA are higher at 285 nm, the selectivity is lower. Method D produces samples having the fewest interferences. Peaks: 1 = N^5-methyl-THFA; 2 = CF; 3 = MTX.

Method D yields the leanest samples. Chromatograms of samples from patients are shown in Fig. 5.

The day-to-day precision obtained with method C was 3.1, 7.0 and 3.5% for N^5-methyl-THFA, CF and MTX, respectively, and that obtained with method D is 5.0 and 6.1% for N^5-methyl-THFA and CF, respectively. With spiked samples a linear calibration graph was obtained up to at least 50 μM for all compounds with method C and for N^5-methyl-THFA and CF with method D. Fig. 6 shows that methods C and D give identical results for the latter compounds.

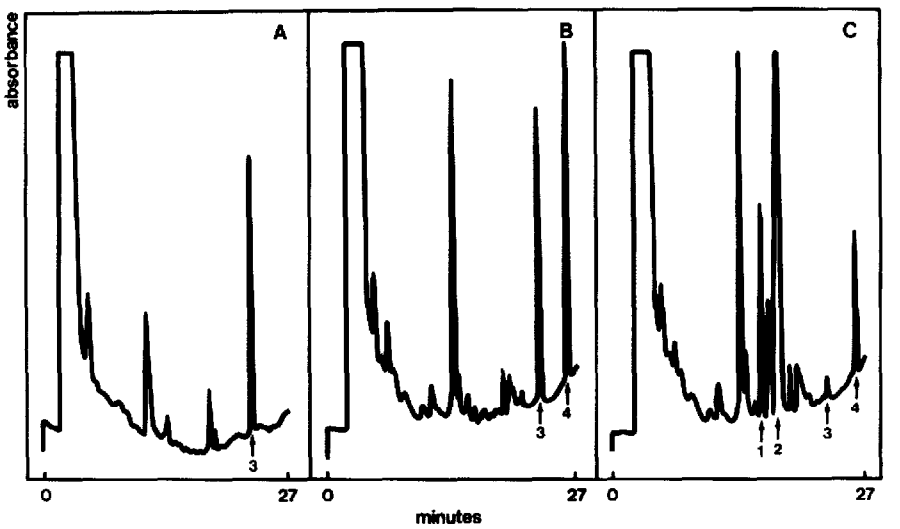

Fig. 5. Chromatograms of samples from patients pretreated according to method C. (A) Cerebrospinal fluid taken 50 h after the patient had received 5 mg of MTX (intrathecally). Only a peak that represents MTX (3) is identified. (B) Plasma sample obtained 24 h ($t=24$ h) following the administration of 225 mg of MTX. MTX (410 nM) (3) and 7-OH-MTX (1050 nM) (4) are present. (C) Plasma sample from the same patient ($t=48$ h). Rescue therapy with CF (50 mg) was started at $t=24$ h. The amount of MTX (3) and 7-OH-MTX (4) present decreased (concentrations 43 and 300 nM, respectively).

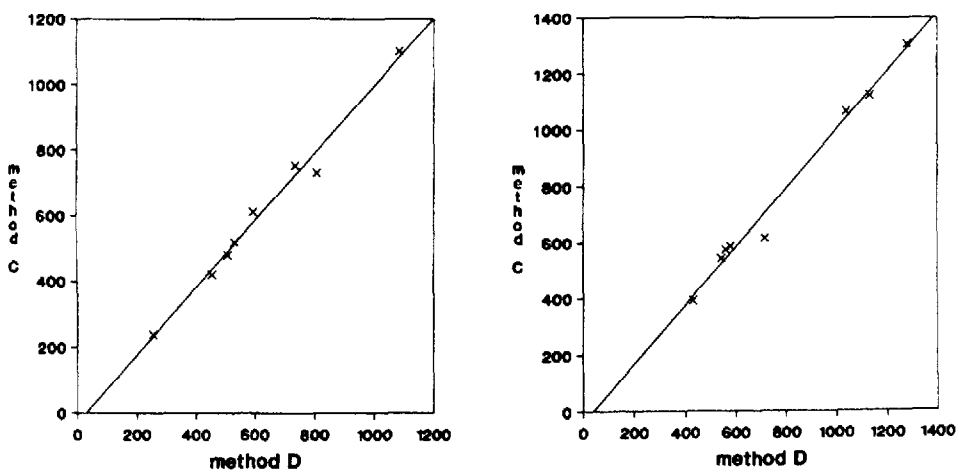

Fig. 6. Comparison of methods C and D. Plasma was drawn from healthy volunteers on two occasions. The concentrations found by both methods show a good correlation, and the regression coefficient and intercept do not differ significantly from 1 and 0, respectively. Left: leucovorin; $y=1.027x-30$, $r=0.993$. Right: N^5-methyl-THFA; $y=1.044x-0.41$, $r=0.993$.

DISCUSSION

The chromatographic conditions used in this study are based on the procedures reported by Cairnes and Evans [4]. Modification of the course of the gradient

enabled us to determine MTX, 7-OH-MTX, CF and N^5-methyl-THFA in the same run.

N^5-Methyl-THFA and CF are reported to be unstable compounds owing to oxidation [6–8]. This study shows that deproteinization with TCA leads to rapid conversion of CF. No method could be found to improve its stability in the presence of TCA. Method D was expected to give the most stable samples, as no oxidative reagents are used. However, it appeared inferior to method C. The stability of the pretreated samples obtained by both methods C and D is improved when they are stored at 4°C prior to analysis, but this procedure requires an autosampler provided with a cooled sample tray or manual sample injection. Alternatively, if large amounts of ascorbic acid are added to the pretreated samples, method C provides samples that are sufficiently stable at room temperature to permit the chromatographic analyses to be performed overnight. Samples obtained with method D must be analysed within 4 h.

With this HPLC method, the detection of minimal amounts of CF and N^5-methyl-THFA is not limited by the signal-to-noise ratio of the chromatographic system but by the presence of interfering endogenous compounds. Preconcentration on top of a narrow-bore (3 mm I.D.) analytical column followed by sequential elution by means of a gradient permits the injection of a relatively large sample volume without affecting the chromatographic performance. If the solution does not differ too much from the mobile phase composition with respect to pH (3.5 ± 0.5) and organic modifier present ($< 5\%$ acetonitrile), up to at least 1 ml can be injected. The selectivity for CF and N^5-methyl-THFA is greatly enhanced if detection is performed at 305 nm rather than 285 nm, which is the optimum absorbance wavelength for CF. Although method D [using 2 ml of acetonitrile–buffer (5:95)] yields samples containing less interfering compounds, the accurate determination of N^5-methyl-THFA and CF down to 200 n*M* will in most instances be possible using method C. This is sufficient for monitoring plasma levels following administration of CF. Samples that contain interfering compounds can be analysed using method D.

In our experience (with about 1000 samples), the detection limit for MTX in samples pretreated using method C (100 μl injected) is less then 50 n*M*, which implies that the method is suitable for monitoring plasma levels following MTX therapy. In two patients, co-eluting compounds of unknown origin interfered with the determination of MTX. This problem could be solved by repeating the measurement at 350 nm instead of 305 nm. The loss of sensitivity [the molar absorption ratio (350 nm/305 nm) for MTX at pH 3.5 is 0.30] can be reduced by injecting more sample. The satisfactory results for MTX and its 7-hydroxy metabolite obtained by using method C have eliminated the need to optimize the solid-phase extraction method for their determination.

In conclusion, a sensitive and stable HPLC method has been designed for the simultaneous determination of N^5-methyl-THFA, CF, MTX and 7-OH-MTX. Samples can be pretreated using a rapid and inexpensive method. A more laborious but selective solid-phase extraction method can be used for samples containing only minimal amounts of CF and/or N^5-methyl-THFA.

REFERENCES

1 R.G. Stoller, K.R. Hande, S.A. Jacobs, S.A. Rosenberg and B.A. Chabner, N. Engl. J. Med., 297 (1977) 630.
2 V. Raso, Cancer Treat. Rep., 61 (1977) 585.
3 E. Scheufler, Clin. Chim. Acta, 111 (1981) 113.
4 D.A. Cairnes and W.E. Evans, J. Chromatogr., 231 (1982) 103.
5 S. Eksborg and H. Ehrsson, J. Chromatogr., 340 (1985) 31
6 B. Payet, G. Fabre, N. Tubiana and J.P. Cano, Cancer Chem. Pharm., 19 (1987) 319.
7 J. Lankelma and E. van der Kleijn, J. Chromatogr., 182 (1980) 35.
8 B.K. Birmingham and D.S. Greene, J. Pharm. Sci., 72 (1983) 1306.