CHROMBIO. 1286

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF METHOTREXATE, 7-HYDROXYMETHOTREXATE, 4-DEOXY-4-AMINO-N10-METHYLPTEROIC ACID AND SULFAMETHOXAZOLE IN SERUM, URINE AND CEREBROSPINAL FLUID

DAVID A. CAIRNES and WILLIAM E. EVANS*

Clinical Pharmacokinetics Laboratory, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101 (U.S.A.)

SUMMARY

An automated high-performance liquid chromatographic system is described for separation and quantitation of the antineoplastic drug methotrexate and metabolites, and the antibiotic sulfamethoxazole in body fluids. The 40-min analysis utilizes a reversed-phase C_{18} column and gradient elution with detection by absorbance of ultraviolet light at 308 nm. The minimum detectable quantities with this assay are: methotrexate 4.4 ng ($9.8 \cdot 10^{-12}$ mole); 4-deoxy-4-amino-N^{10}-methylpteroic acid 11.9 ng ($3.7 \cdot 10^{-11}$ mole); 7-hydroxymethotrexate 30 ng ($6.5 \cdot 10^{-11}$ mole); sulfamethoxazole 125 ng ($4.9 \cdot 10^{-10}$ mole). This analytical method should prove useful for therapeutic monitoring and pharmacokinetic studies of these compounds.

INTRODUCTION

Methotrexate, L-(+)-N-(p-[((2,4-diamino-6-pteridinyl)methyl)methylamino]-benzoyl)glutamic acid, a competitive inhibitor of dihydrofolate reductase, belongs to the first class of compounds successfully used to produce remission of leukemia in man. Methotrexate (MTX) is now widely used to treat several human cancers including acute leukemia, osteosarcoma, non-Hodgkin's lymphoma, breast carcinoma and choriocarcinoma.

Renal excretion is the major route of MTX and metabolite elimination. Depending on the dosage and duration of intravenous (i.v.) infusion, from 60—95% of an i.v. dose of MTX may be eliminated unchanged in the urine. More recent pharmacokinetic studies [1,2] have identified substantial amounts of 7-hydroxymethotrexate (7-OH MTX), an oxidative metabolite formed by hepatic aldehyde oxidase [3]. Since 7-OH MTX has 3—5 times lower solubility

than the parent compound, intratubular crystallization of this metabolite has been proposed as one mechanism of renal damage following MTX administration [3]. MTX may also be metabolized to 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) via a simple amide cleavage by intestinal bacteria [4]. Both of these metabolites are about 100—200-fold less cytotoxic than MTX [4,5].

Because the cytotoxic effects of MTX are due to the inhibition of dihydrofolate reductase and the subsequent lowering of reduced folate pools, the effects of MTX can be negated by administering reduced folates (i.e., leucovorin "rescue"). This has provided a mechanism by which dosage of MTX (1—20 g/m^2) far in excess of conventional dosages (25—50 mg/m^2 without leucovorin) can be given without host toxicity. The rationale of these higher dosages includes overcoming relative resistance due to poor intracellular uptake of MTX and achieving cytotoxic MTX concentrations in tissues where MTX distribution is poor (i.e., central nervous system, testes). However, the administration of high-dose MTX may be severely toxic if adequate leucovorin rescue is not given. A nationwide survey showed approximately a 6% incidence of drug-related deaths after high-dose MTX and leucovorin rescue [6]. This noted mortality rate solidified the need for close clinical and pharmacokinetic monitoring of high-dose MTX and leucovorin rescue.

Several protocols have been described for monitoring high-dose MTX and adjusting leucovorin rescue in patients with delayed MTX elimination [7]. All of these protocols include measurement of MTX serum or plasma concentrations to identify high-risk patients and to guide the dosage modification of leucovorin. Several pharmacokinetic studies have also been conducted to more precisely define the influence of selected clinical features (i.e., pleural effusions, ascites, gastrointestinal obstruction, renal dysfunction, etc.) on the delayed elimination of MTX. However, most studies have not examined the contribution of altered MTX metabolism or perturbations in MTX disposition induced by metabolites of MTX. The absence of such studies is related in part to the lack of an accurate, sensitive and clinically feasible assay for MTX and its two major metabolites in biological fluids. These pharmacokinetic studies are also frequently complicated by the concomitant administration of sulfamethoxazole—trimethoprim, a drug combination which is routinely used as prophylaxis for *Pneumocystis carinii* pneumonia in immunosuppressed patients at high-risk for this potentially fatal infection. The presence of sulfamethoxazole [N'-(5-methyl-3-isoxazoyl)sulfanilamide] in biologic fluids being assayed for MTX and metabolites is potentially important, since this compound co-elutes with MTX metabolites on some high-performance liquid chromatographic (HPLC) systems and because sulfamethoxazole may influence the pharmacokinetics of MTX (i.e., protein binding, renal tubular secretion).

Several assay methods for MTX have been reported and include enzyme immunoassay (EMIT) [8,9], radioimmunoassay [10—14], radioenzymatic [13,15] and HPLC assays [16—20]. Of these techniques, only HPLC has the potential to readily measure both parent drug and metabolites. To date, an HPLC assay which provides separation and quantitation of MTX, DAMPA, 7-OH MTX and sulfamethoxazole (SMX) has not been reported.

EXPERIMENTAL

Reagents

Water was distilled and treated with a Milli-Q water purification system. UV grade acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and certified ammonium formate (Fisher, Pittsburgh, PA, U.S.A.) were used as received. Reagent grade trichloroacetic acid was obtained from VWR Scientific (San Francisco, CA, U.S.A.). Ethanol 95% U.S.P. (AAper Alcohol and Chemical Co., Louisville, KY, U.S.A.) was used as received. HPLC solvent A was 5% acetonitrile in 0.01 M ammonium formate solution, HPLC solvent B was 20% acetonitrile in 0.01 M ammonium formate solution (pH 3.5 for both eluting solvents). HPLC solvents were purged with helium and subjected to vacuum to degas. SMX and aminopterin (AMN) were purchased from Sigma (St. Louis, MO, U.S.A.). DAMPA and 7-OH MTX were gifts from Dr. D. Kabbakoff. DAMPA was also synthesized as described below. MTX, prepared as the sodium salt, NCI No. NSC-740 was supplied by the National Cancer Institute. Structures of the compounds of interest are shown in Fig. 1.
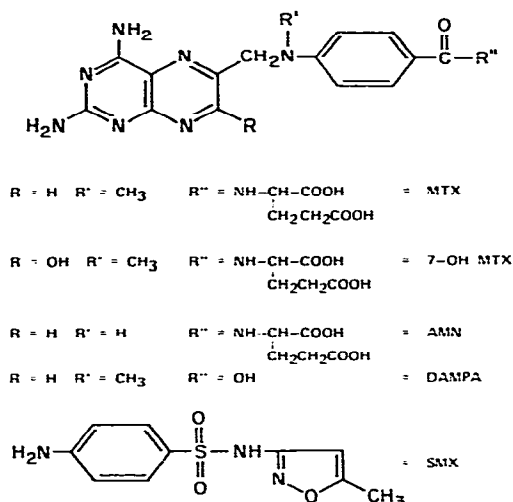

Fig. 1. Chemical structures of methotrexate (MTX), 7-hydroxymethotrexate (7-OH MTX), aminopterin (AMN), 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and sulfamethoxazole (SMX).

Synthesis of DAMPA

Amides are cleaved to carboxylic acid salts with aqueous bases, acidification gives the actual acid. To 20 ml 0.05 N sodium hydroxide were added 60 mg MTX, and the solution was refluxed overnight. Conversion of MTX to DAMPA was about 50% as determined by UV absorbance with no side products. The solution was neutralized or slightly acidified with hydrochloric acid and rotary evaporated under vacuum. DAMPA was isolated and MTX recovered easily in small amounts by using the HPLC system described.

Samples

Calibration samples were prepared from pooled normal human serum spiked with the compounds of interest and serially diluted. To 0.5 ml of sample were added 5 μl (250 ng) of AMN as internal standard, with vortexing. Proteins were precipitated by adding 75 μl 2 *M* trichloroacetic acid in 95% ethanol and vortexing. Centrifugation at 1500 *g* for 15 min yielded a clear supernatant suitable for HPLC injection. A volume of 250 μl was auto-injected on a 200-μl loop.

Blood, cerebrospinal fluid (CSF), and urine were collected at various times from patients receiving MTX (200 mg/m² i.v. bolus, followed by 800 mg/m² infused i.v. over 24 h). These patients also received an intrathecal MTX dose of 12 mg/m² at the start of the 24-h infusion (time = 0 h). Blood was centrifuged and serum was removed by pipet for analysis. Urine was diluted 1:10 with buffer A. CSF was submitted for analysis as collected. All samples were otherwise treated exactly as the calibration standards.

Instrumentation

HPLC was performed on a prepacked μBondapak 10-μm C_{18} column 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.) protected with a 2.3 cm × 3.9 mm precolumn packed with C_{18} Corasil, 37—50 μm (Waters Assoc.). An M-45 HPLC pump (Waters Assoc.) augmented with a 980A solvent programmer (Tracor Instruments) was used as the solvent delivery system. Gradient elution was utilized for optimum resolution with the following parameters: 3.3% change per min, 20% non-linear convex curve, 25—100% B, flow-rate 2.0 ml/min. Samples were handled by a Model 8055 Autosampler (Varian) fitted with an AH-60 pneumatic injector (Valco Instruments). Column effluent was detected by a Model 1203 UV III monitor (Laboratory Data Control) illuminated with a zinc lamp at a fixed wavelength of 308 nm. A sensitivity of 0.002 a.u.f.s. was used. Detector signal data were processed with an S/P-4100 computing integrator (Spectra Physics). Retention times and areas of peaks calculated by the SP-4100 were stored on tape by a D-980M Cassette Tap Deck (Hitachi) for possible reprocessing.

RESULTS AND DISCUSSION

Of several solvent system—column packing combinations investigated, the system described (see Experimental) proved convenient and satisfactory, achieving baseline resolution for all components of interest. Separation of a standard mixture in pooled serum is shown in Fig. 2, along with a chromatogram from a serum blank. Retention volumes and capacity factors for the compounds of interest and other folates are given in Table I. Leucovorin (5-formyl-tetrahydrofolate) and its metabolite 5-methyl-tetrahydrofolate (5-MeTHF), which may be present in patient samples after leucovorin rescue is begun, are resolved from the compounds of interest.

Quantitative estimation of the amounts of parent drug and/or metabolites was obtained with this system. Calibration curve data with internal standard correction were processed by the computing integrator calculating a linear fit to detector response. The linear correlation coefficients were: MTX 0.9898, DAMPA 0.9575, SMX 0.9965, 7-OH MTX 0.9956. The minimum detectable
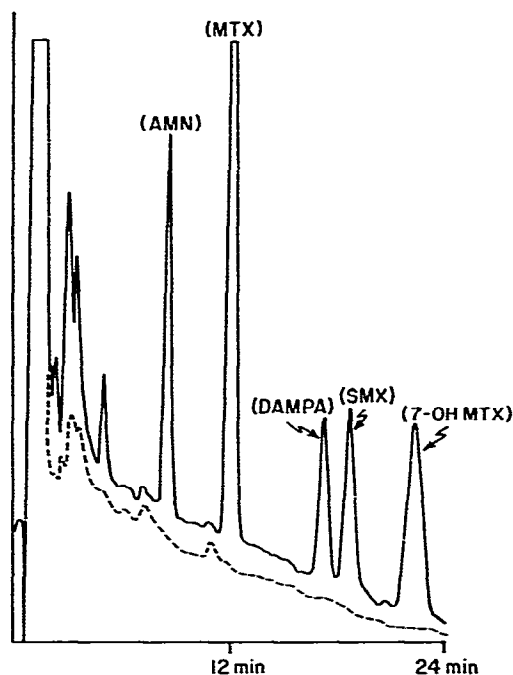
Fig. 2. Chromatogram of blank serum (———) and serum spiked with known standards (———). Abbreviations as given in Fig. 1, recorded attenuation 32 mV full scale.

TABLE I

RETENTION VOLUMES AND CAPACITY FACTORS FOR THE COMPOUNDS INVESTIGATED

Compound	Retention volume (ml)	Capacity factor (k')
5-MeTHF	11.3	3.5
AMN	17.0	5.1
Leucovorin	19.3	6.7
MTX	23.3	7.3
Folic acid	24.9	9.0
DAMPA	30.6	9.9
SMX	35.0	11.5
7-OH MTX	43.3	14.5

quantities with this assay were calculated as: MTX 4.4 ng ($9.8 \cdot 10^{-12}$ mole), DAMPA 11.9 ng ($3.7 \cdot 10^{-11}$ mole), 7-OH MTX 30 ng ($6.5 \cdot 10^{-11}$ mole), SMX 125 ng ($4.9 \cdot 10^{-10}$ mole) and found to be in agreement with our experimental values. The technique can thus easily detect and measure quantities of these molecules in the nanogram range. The ultimate usefulness of the method is in detecting the presence of MTX and metabolites in patient body fluids and conducting pharmacokinetic studies of MTX and its metabolites. The minimum detectable concentration of samples prepared as described in the experimental procedure proved to be: MTX $4.8 \cdot 10^{-8}$ M, DAMPA $1.8 \cdot 10^{-7}$ M, 7-OH MTX $3.2 \cdot 10^{-7}$ M and SMX $2.5 \cdot 10^{-6}$ M. Obviously, with an added technique of sample
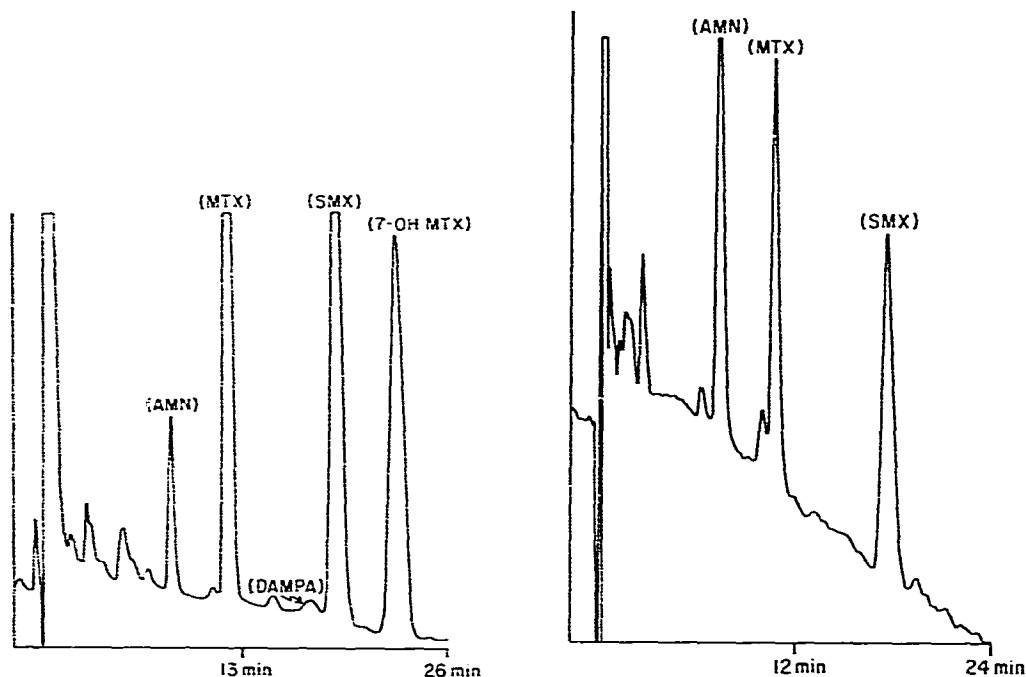
Fig. 3. Chromatogram of a patient serum sample obtained 25 h after starting a 24-h i.v. infusion of MTX (200 mg/m² i.v. bolus, 800 mg/m² infused over 24 h). SMX, 20 mg/kg had been given orally 6 h prior to collection of this serum sample. Abbreviations as given in Fig. 1, recorded attenuation 64 mV full scale.

Fig. 4. Chromatogram of a patient CSF sample obtained 24 h after starting a 24-h i.v. MTX infusion (see Fig. 3 for dosage). An intrathecal dose of MTX (12 mg/m²) had been given at the start of the MTX infusion and a dose of SMX (20 mg/kg) had been given orally 6 h prior to collection of this CSF sample. Abbreviations as given in Fig. 1, recorded attenuation 16 mV full scale.

preconcentration lower concentrations can be quantitated. For our pharmacokinetic studies, the minimum detectable concentrations achieved were sufficient.

As a measure of system precision, 16 replicate injections ($5 \cdot 10^{-6}$ M MTX, $0.4 \cdot 10^{-6}$ M DAMPA, $0.7 \cdot 10^{-6}$ M 7-OH MTX) were performed under assumed identical conditions. The coefficients of variation (C.V.) for MTX, DAMPA and 7-OH MTX concentrations were 9.7%, 12.7% and 13.7%, respectively.

Sample preparation was fast, simple and reproducible. Total analysis time including gradient return and column re-equilibration was 40 min. Representative chromatograms of patient serum, CSF, and urine are shown in Figs. 3, 4 and 5, respectively. Note from Fig. 4 there were no apparent metabolites of MTX in CSF, and SMX readily crossed the blood—CSF barrier. In these patients, MTX was administered as an initial 200 mg/m² i.v. bolus followed by an 800 mg/m² i.v. infusion over 24 h. The intrathecal MTX dosage was 12 mg/m². SMX, 20 mg/kg body weight, was given orally every 12 h. In Fig. 5, peaks X_1 and X_2 are presently unidentified components which were not seen in all urine samples and do not appear to be due to other drugs being administered to these patients.
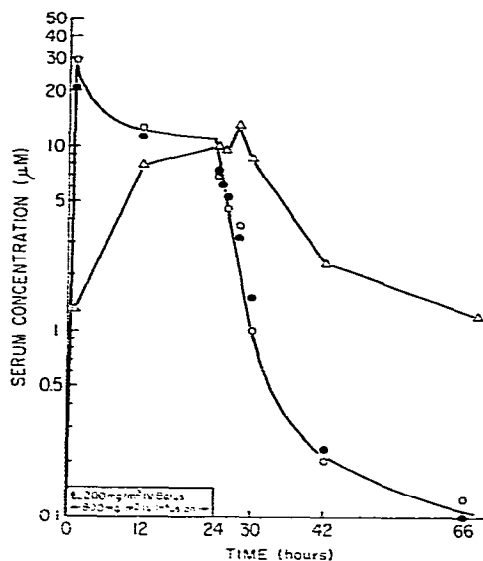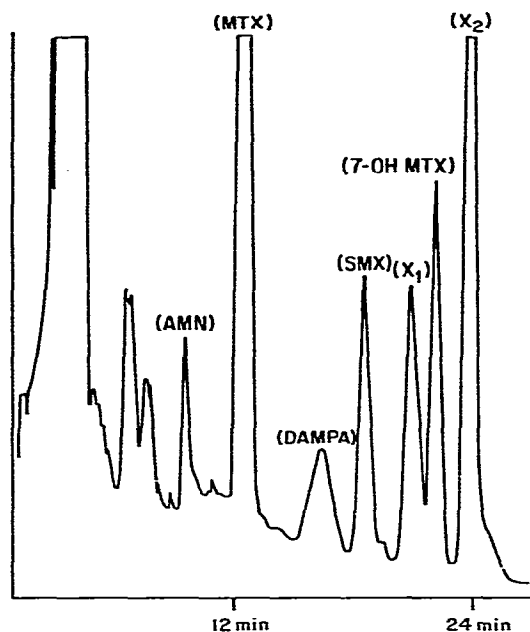
Fig. 5. Chromatogram of a patient urine sample collected 0—48 h after starting a 24-h i.v. MTX infusion (see Fig. 3 for dosage). SMX, 20 mg/kg was also given orally every 12 h during this interval. Abbreviations as given in Fig. 1, recorder attenuation 64 mV full scale.

Fig. 6. Serum concentration—time profiles for MTX (o, HPLC; ●, EMIT) and 7-OH MTX (△, HPLC) from a representative patient. DAMPA was not detected in serum.

Serum concentration—time profiles for MTX and 7-OH MTX, obtained from a representative patient, are shown graphically in Fig. 6. DAMPA was generally not detected in serum or CSF, and only in low concentrations in urine. It is possible that the antibacterial effects of SMX may have affected the intestinal bacteria responsible for metabolizing MTX to DAMPA.

CONCLUSION

For the analysis of body fluids, a sensitive HPLC method should be fast, selective and have a wide linear dynamic range. The procedure described represents such a method for quantitating MTX, DAMPA, 7-OH MTX, and SMX. The technique is simple, easily automatable and inexpensive to operate. Measurement of parent drugs and metabolites at times 66 h post infusion can be achieved.

REFERENCES

1 R. Buice, W.E. Evans, J.C. Karas, C.A. Nicholas, A. Straughn, M.C. Meyers and W.R. Crom, Clin. Chem., 26 (1980) 1902—1904.
2 C. Canfell and W. Sadee, Cancer Treat. Rep., 64 (1980) 165—169.
3 S.A. Jacobs, R.G. Stoller, B.A. Chabner and D.G. Johns, J. Clin. Invest., 57 (1976) 534—538.
4 D.M. Valerino, D.G. John, D.S. Zaharko and V.T. Oliverio, Biochem. Pharmacol., 21 (1972) 821—831.
5 D.G. Johns and T.L. Loo, J. Pharm. Sci., 56 (1967) 356—359.
6 O.D. VonHoff, J.S. Penta, L.J. Helman and M. Slavik, Cancer Treat. Rep., 61 (1977) 745—748.
7 W.E. Evans, in W.E. Evans, J.J. Schentag and W.J. Jusko (Editors), Applied Pharmaco-kinetics, Applied Therapeutics Inc., San Francisco, CA, 1980, p. 518.
8 C. DePorcer-Morton, J. Chang, M. Specker and R. Bastiani, Performance Evaluation of EMIT® Methotrexate Assay, Syva Co., Palo Alto, CA, November 1978.
9 J.B. Gushaw and J.G. Miller, Clin. Chem., 24 (1978) 1032.
10 J. Raso and R. Schreiber, Cancer Res., 35 (1975) 1407—1410.
11 J. Hendel, L.J. Sarek and E.F. Hvidberg, Clin. Chem., 22 (1976) 813—816.
12 Y. Wang, E. Lantin and W.W. Sutow, Clin. Chem., 22 (1976) 1053—1056.
13 Methotrexate ^{125}I RIA Kit Instruction Manual, Diagnostic Biochemistry Inc., San Diego, CA, June 1978.
14 G.W. Aherne, E.M. Piall and J. Marks, Brit. J. Cancer, 36 (1977) 608.
15 L.C. Falk, D.R. Clark, S.M. Kalman and T.F. Long, Clin. Chem., 22 (1976) 785—788.
16 G.J. Lawson, P.F. Dixon and G.W. Aherne, J. Chromatogr., 223 (1981) 225—231.
17 W.P. Tong, J.L. Wisnick, J. Horton and D.B. Ludlum, Clin. Chim. Acta, 107 (1980) 67—72.
18 E. Watson, J.L. Cohen and K.K. Chan, Cancer Treat. Rep., (1978) 381—387.
19 J.L. Cohen, G.H. Hisayasu, A.R. Barrientos, M.S.B. Nayar and K.K. Chan, J. Chromatogr., 181 (1980) 478—483.
20 J.A. Nelson, B.A. Harris, W.J. Decker and D. Farquhar, Cancer Res., 37 (1977) 3970.