Journal of Chromatography, 413 (1987) 320–325
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3393

Note

Rapid and simple assay for the measurement of methotrexate in serum, urine and red blood cells by reversed-phase high-performance liquid chromatography

PAUL A. BRIMMELL* and DAVID J. SAMS

Department of Biochemistry, Royal Sussex County Hospital, Eastern Road, Brighton, BN2 5BE Sussex (U.K.)

(First received May 29th, 1986; revised manuscript August 19th, 1986)

Methotrexate (MTX; 4-amino-N^{10}-methylpteroyl-L-glutamic acid) is a structural analogue of folic acid. It binds tightly to dihydrofolate reductase (DHFR), the enzyme responsible for the conversion of folic acid to reduced folate co-factors [1,2].

Folate antagonists, and particularly MTX, have played an increasingly important role in cancer chemotherapy since their introduction into clinical practice in 1948 [3], when small doses were used. In recent years, however, massive doses, as high as 30 000 mg/m^2 with rescue treatment [1], have been administered. The value of MTX is now established in the treatment of acute lymphocytic leukaemia, non-Hodgkin's lymphoma, choriocarcinoma, breast cancer, osteosarcoma, small cell lung cancer, head and neck cancer and intrathecal chemotherapy. It may also be applied in non-malignant conditions such as severe psoriasis. However, high-dose MTX therapy is accompanied by acute folate stress, which can lead to the death of the patient [4]. The toxic effects include bone marrow depression, renal failure, hepatotoxicity and pulmonary problems.

Because the cytotoxic effects of MTX are due to the inhibition of DHFR and the subsequent lowering of reduced folate pools, the effects of MTX can be negated by administering reduced folates (e.g. leucovorin 'rescue') [5]. Owing to the inherent risk of toxicity from this regime, optimal patient management necessitates the monitoring of plasma MTX.

There are many analytical methods in the literature for plasma MTX including enzyme inhibition, competitive protein binding, enzyme multiplied immunoassay (EMIT) [6], radioimmunoassay [7,8], microbiological, fluorimetry and high-

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

performance liquid chromatography (HPLC) [9–20]. Of these techniques only HPLC has the potential to readily measure both the parent drug and metabolites, the most important of which, 7-hydroxymethotrexate (7-OHMTX), may cause renal damage due to crystallization in the tubules [21].

The method described in this paper has the advantage of requiring a small sample, only 225 μl of plasma, compared to the 0.5–1.0 ml needed by most of the other published methods, without loss of sensitivity. The total analysis time of less than 15 min per specimen is possibly the fastest so far recorded. This assay is also suitable for red blood cell MTX measurements. This field has attracted very little interest thus far, the much longer procedure of Farid et al. [9] being one of the few.

EXPERIMENTAL

Equipment

A Pye-Unicam LC-XPD HPLC pump (Pye-Unicam, Cambridge, U.K.), a Cecil CE212 variable-wavelength UV monitor (Cecil Instruments, Cambridge, U.K.) and a Vitatron UR403 single-pen recorder (Scientific Instruments, Crawley, U.K.) were used. The column (100×4.5 mm I.D.) was obtained from Anachem (Luton, U.K.) and packed in our laboratory with 5-μm ODS-Hypersil using a hydraulic pump (Standsted Fluid Power, Crawley, U.K.) and a stainless-steel reservoir (Biomedical Engineering Department, Sussex University, Brighton, U.K.). Injections were performed with a 10-μl Hamilton syringe (V.A. Howe, London, U.K.).

The samples were prepared in Beckman microtubes (Beckman, High Wycombe, U.K.) with centrifugation in an Eppendorf microtube centrifuge (Anderman, Kingston-upon-Thames, U.K.). Mobile phase preparation involved the use of an ultrasonic bath (Baird and Tatlock, Romford, U.K.).

Reagents

MTX and aminopterin (4-aminofolic acid), used as internal standard, were obtained from Sigma (Poole, U.K.). Tetrahydrofuran was HPLC grade (Quadrachem Labs., Forest Row, U.K.). All other reagents were obtained from British Drug Houses (Poole, U.K.).

Procedure

A 25-μl volume of internal standard (aminopterin 40 mg/l in water, with 200 μl of 1 *M* sodium hydroxide added to aid solution) was added to 225 μl plasma in a Beckman microtube, to allow centrifugation in an Eppendorf microcentrifuge (at 9980 *g*). Acetone (250 μl) was added as protein precipitant and mixed using a vortex mixer. The mixture was then centrifuged for 30 s in the Eppendorf microcentrifuge. A volume of 300 μl of the supernatant was transferred to a second microtube containing 300 μl butan-1-ol and 400 μl diethyl ether. This was again mixed using the vortex mixer, then centrifuged for 30 s in the microfuge. The supernatant was discarded, then 10 μl of the remaining solution injected onto the column.

The mobile phase, which was 4% tetrahydrofuran in 0.05 M sodium dihydrogen orthophosphate buffer, was run at 0.6 ml/min. The mobile phase was filtered using a Whatman glass microfibre filter paper, then degassed by immersion in an ultrasonic bath for 5 min. Chromatography was performed at ambient temperature with detection at 305 nm. MTX concentrations were determined by peak-height comparisons with a standard curve, prepared daily by diluting a stock MTX standard with drug-free pooled plasma to give a standard range from $0.22 \cdot 10^{-5}$ M to $4.4 \cdot 10^{-5}$ M.

Red blood cell MTX assay

A simple, reliable assay was also required for the measurement of MTX in human red blood cells, in order that studies be performed on membrane characteristics of the drug. Using the same procedure as described for plasma MTX, it was not possible to obtain 300 μl of supernatant from 225 μl of haemolysate and added protein precipitant. Also, the final sample volume for injection after mixing with diethyl ether and butan-1-ol was much smaller than that obtained from plasma. The volumes and procedure used were therefore modified.

The red blood cells were washed three times with an equal volume of isotonic saline. After the third wash a haemolysate was formed by the addition of an equal volume of deionised water.

After thorough mixing, a 500-μl volume of haemolysate was taken, to which were added 50 μl of internal standard and 550 μl acetone. The mixture was mixed and then centrifuged. The supernatant was treated as described for the plasma estimation except that 15 μl of the mobile phase were added to the final injection mixture. After careful mixing 10 μl were injected onto the column.

RESULTS AND DISCUSSION

Chromatograms from blank human plasma, plasma spiked with MTX and internal standard, and plasma from a patient on MTX therapy are shown in Fig. 1.

The retention times for MTX and internal standard were 4 and 2.5 min, respectively. The recovery of MTX from pooled plasma estimated by comparison with aqueous standards was 62.7%. The mean within-batch coefficient of variation was 5.6%. This was determined by spiking pooled plasma with MTX and assaying a minimum of ten times during a single run, and repeating the run on ten different days. The between-batch coefficient of variation was 15.9%. This latter figure is almost certainly falsely high due to a degree of column deterioration over the period of time during which the precision testing was done. The column was repacked during this period.

The on-column limit of sensitivity for MTX was $6.6 \cdot 10^{-8}$ M, at which concentration the peak height was three times the noise level of the system. The minimum detectable concentration of samples prepared as described in the experimental procedure was $1.1 \cdot 10^{-7}$ M. Table I lists some of the drugs commonly co-administered with MTX that have so far been tested on this system.

None of the drugs tested produced any interfering peaks on the chromatogram.

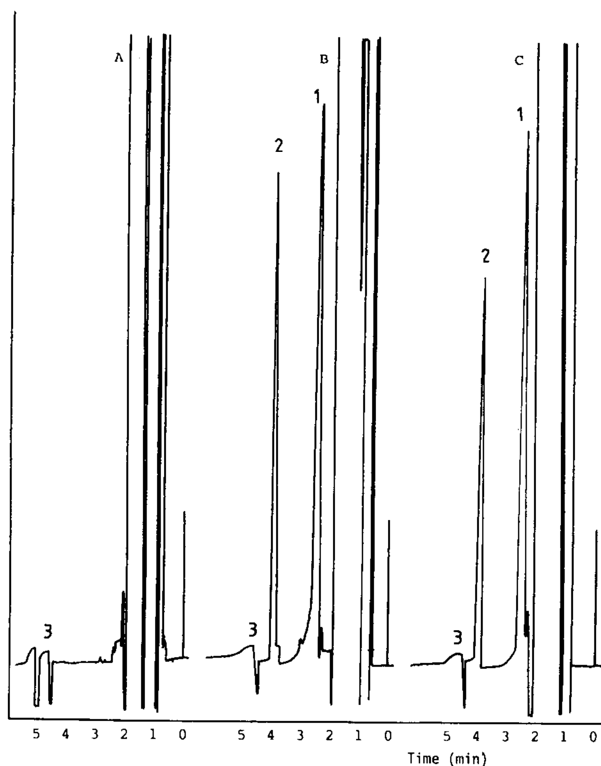

Fig. 1. Chromatograms for (A) blank human plasma, (B) plasma spiked with MTX and internal standard and (C) plasma from a patient on MTX therapy to which internal standard has been added. Peaks: 1 = internal standard (aminopterin); 2 = MTX; 3 = interference effect of butan-1-ol and diethyl ether. Time 0 marks the point of injection. Detector sensitivity setting was 0.01 a.u.f.s. and recorder chart speed was 0.5 cm/min.

A good correlation was obtained against an established EMIT assay. The linearity of the system was determined by running standard curves giving an MTX concentration range from 0 (drug-free pooled plasma) to $2.2 \cdot 10^{-5} M$. The system was found to be linear over this concentration range.

Fig. 2 illustrates a typical chromatogram for a red blood cell haemolysate spiked with MTX and internal standard. The retention times for red blood cell MTX and internal standard were the same as achieved in the procedure for plasma

TABLE I

DRUGS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Caffeine	Fluorouracil	Nitrazepam
Chlordiazepoxide	Folinic acid	Phenobarbitone
Cortisol	Hydralazine	Prednisolone
Cyclophosphamide	Imipramine	Salicylate
Desipramine	Mercaptopurine	Trimethoprim
Dexamethasone	Morphine	Trimipramine
Diazepam		

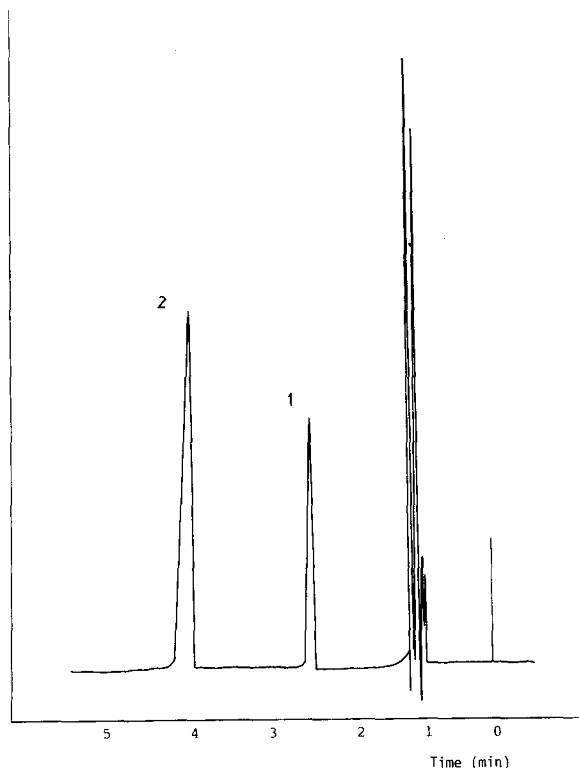

Fig. 2. Chromatogram from a haemolysate spiked with MTX and internal standard. Peaks: 1 = internal standard (aminopterin); 2 = MTX. Time 0 marks the point of injection. Detector sensitivity setting was 0.05 a.u.f.s. and recorder chart speed was 0.5 cm/min.

levels. The minimum detectable concentration of MTX in red blood cells was $5.5 \cdot 10^{-8}\,M$. The mean within-batch coefficient of variation was 7.7%. No interfering peaks have been observed on the chromatograms.

Of several solvent systems investigated, the system described in this paper proved to be convenient and satisfactory. It would appear to be ideal for the routine biochemistry laboratory with a heavy workload. Sample preparation was rapid, a batch of six samples being ready for injection in little over 5 min. The method is simple, employing few steps. Within-batch precision is very good. This method also has the advantage of a short chromatography time, enabling the average laboratory worker to analyse at least six samples per hour. The sensitivity is great enough to monitor patients receiving MTX therapy, but could, if desired, be increased by scaling up the volumes used in the experimental procedure and injecting an increased sample volume by using a valve injection system. The system can be used to monitor urine MTX levels but with some patient samples is susceptible to interference from substances not removed by the simple sample preparation stage.

The materials used were all common and relatively inexpensive. This, coupled with the speed and reliability of the assay, provides an excellent choice of method in the present economic climate.

REFERENCES

1 W.A. Bleyer, Cancer, 41 (1978) 36-51.
2 B.A. Chabner, R.C. Donehower and R.L. Schilsky, Cancer Treat. Rep., 65 (Suppl. 1) (1981) 51-54.
3 S. Farber, L.K. Diamond, R.D. Mercer, R.F. Sylvester and J.A. Wolff, N. Eng. J. Med., 238 (1948) 787-793.
4 D.D. Von Hoff, J.S. Penta, L.J. Helman and M. Slavik, Cancer Treat. Rep., 61 (1977) 745-748.
5 W. Sadee, in G. Tognoni, R. Latini and W.J. Jusko (Editors), Frontiers in Therapeutic Drug Monitoring, Raven Press, New York, 1980, pp. 163-171.
6 P.R. Finley, R.J. Williams, F. Griffith and D.A. Lichti, Clin. Chem., 26 (1980) 341-343.
7 J. Mendel, L.J. Sarek and E.F. Hvidberg, Clin. Chem., 22 (1976) 813-816.
8 R. Kamel, J. Landon and G.C. Forrest, Clin. Chem., 26 (1980) 97-100.
9 Y.Y.Z. Farid, I.D. Watson and M.J. Stewart, J. Pharm. Biomed. Anal., 1 (1983) 55-63.
10 K.K. Chan, J.L. Cohen, E. Watson, E. Rubinstein and L. Irwin, in G. Hawk (Editor), Biological/Biomedical Applications of Liquid Chromatography, Marcel Dekker, New York, 1979, pp. 585-602.
11 G.J. Lawson, P.F. Dixon and G.W. Aherne, J. Chromatogr., 223 (1981) 225-231.
12 M.-L. Chen and W.L. Chiou, J. Chromatogr., 226 (1981) 125-134.
13 J.L. Cohen, G.H. Hisayasu, A.R. Barrientos, M.S. Nayar and K.K. Chan, J. Chromatogr., 181 (1980) 478-483.
14 R.G. Buice and P. Sidhu, J. Pharm. Sci., 71 (1981) 74-77.
15 J. Salamoun and J. František, J. Chromatogr., 378 (1986) 173-181.
16 F. Palmisano, T.R.I. Cataldi and P.G. Zambonin, J. Chromatogr., 344 (1985) 249-258.
17 D.A. Cairnes and W.E. Evans, J. Chromatogr., 231 (1982) 103-110.
18 N. So, D.P. Chandra, I.S. Alexander, V.J. Webster and D.W. O'Gorman-Hughes, J. Chromatogr., 337 (1985) 81-90.
19 M. Stout, Y. Ravindranath and R. Kauffman, J. Chromatogr., 342 (1985) 424-430.
20 A. El-Yazigi and C.R. Martin, J. Liq. Chromatogr., 7 (1984) 1579-1591.
21 S.A. Jacobs, R.G. Stoller, B.A. Chabner and D.G. Johns, J. Clin. Invest., 57 (1976) 534-538.