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Comparison of a New High-Performance Liquid Chromatography Method with Fluorescence Polarization Immunoassay for Analysis of Methotrexate

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Summary: A simple high-performance liquid chromatography (HPLC) method for the determination of methotrexate (MTX) in biological fluids is described. The assay is rapid, the time required for analysis is <30 min, and it is sensitive up to 0.01 μg/ml, which is three times below the toxic MTX concentration. Fifty plasma samples drawn from acute lymphocytic leukemia (ALL) patients were used to compare this method with that of fluorescence polarization immunoassay (FPIA). A good correlation (r = 0.979) was obtained between the results of the two analyses. FPIA constantly overestimates the concentration in samples collected during elimination and underestimates those collected during infusion. The difference between the means of the two methods was 29% and 13% for the elimination and infusion samples, respectively. The means of the peak height ratio of the metabolite to MTX in the HPLC chromatograms were 3.39 and 0.33 during elimination and infusion, respectively. The results therefore indicate that HPLC is more specific when tracing the washout of MTX concentration. Because of this specificity and simplicity, the method is recommended for therapeutic drug monitoring. The stability of MTX in human saliva was investigated in this study. MTX was found to be stable at room temperature and at −20°C for a minimum of 3 h and 3 weeks, respectively. **Key Words:** Methotrexate—High-performance liquid chromatography—Fluorescence polarization immunoassay—Acute lymphocytic leukemia—Biological fluids—Stability in saliva.

Methotrexate (MTX) is a primary drug in the treatment of various malignancies, including acute lymphocytic leukemia (ALL) (1,2). The measurement of plasma MTX concentration is now used to detect high-risk patients, as well as to optimise the dose and duration of leucovorin. The beneficial effect of MTX level depends on the ability to measure its plasma concentration rapidly (2,3). The fully automated TDX machines by Abbott [using fluorescence polarization immunoassay (FPIA) principles; Abbott Laboratories, North Chicago, IL, U.S.A.] have recently become available to provide the required speed for analyzing patient samples (2,3). However, because of the problems of expense and selectivity, high-performance liquid chromatography (HPLC) was also considered in clinical analysis. During the past few years, several HPLC methods were developed and applied mainly in research fields (4–9). In this study, we describe a simple and rapid HPLC method that can be used for routine MTX analysis. The assay was compared with the

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most commonly used technique (TDX machines) using plasma samples of ALL patients. Saliva is one of the biological fluids applied in therapeutic drug monitoring (10). In previous studies (11,12), the poor stability of isoniazide and nitrazepam was the primary factor behind the low concentration of these drugs in saliva. Because the salivary concentration of MTX is also known to be low (13), its stability at room temperature and at −20°C was studied.

MATERIALS AND METHODS

Drug Analysis

MTX was measured using a Waters HPLC system (Waters Associates, Milford, MA, U.S.A.). The system consists of a 720 system controller, 730 data module, 481 UV detector, 710 B automatic injector, M45 solvent delivery system, and a reverse-phase Novapak C18 column (4 μm, 10 cm × 8 mm I.D.). To each 250 μl plasma, saliva, or cerebral spinal fluid (CSF), 20 μl of the internal standard (IS) 4-amino-acetophenone was added and then deproteinized by the addition of 40 μl trichloro-acetic acid (2 *M* in ethanol). The mixture was mixed for 1 min and then centrifuged at 3,000 *g* for 15 min. The supernatant (25–100 μl) was then injected onto the HPLC column and eluted by using a phosphate buffer, methanol, and acetonitrile (84:11:5) as the mobile phase. The flow rate was 2.3 ml/min and the effluent was monitored at 313 nm. The phosphate buffer used consisted of monobasic sodium phosphate (0.1 *M*) and Tris(hydroxymethyl)-aminomethane (10 m*M*); the pH was adjusted to 5.75 with phosphoric acid.

Standard curves of MTX concentration versus the peak area ratios were constructed to cover the ranges of 0.01–1.0 and 1.0–100 μg/ml. The concentration of the IS added was 20 μg/ml for the samples that contained ≤1 μg/ml MTX and 60 μg/ml for those that contained ≥1 μg/ml. To determine the coefficient of variations, human plasma was spiked with MTX to get four standards: 0.25, 1.0, 5.0, and 10 μg/ml. The intra-day variations were then determined by injecting each standard eight times.

Clinical Study

Fifty plasma samples were collected during routine follow-up of seven ALL pediatric patients and assayed for MTX by both FPIA (TDX) and HPLC. The dose of MTX received by this group of patients was 200 mg/m^2 as a bolus, followed by 800 mg/m^2 infused over 24 h. The plasma samples were collected before stopping the infusion and during the elimination period. The following drugs were administered concurrently with MTX as part of the patient treatment plan: promethazine, allopurinol, and cytosine arabinoside. Twelve additional samples were collected in four of the patients at later times, between 48–72 h postinfusion, and assayed only with HPLC. The paired *t* test was used to run the statistical analysis.

Stability Study

Saliva was collected by having volunteers chew on a piece of paraffin. The clean saliva after centrifugation was pooled and spiked with MTX to get 5 and 10 μg/ml. The stability of MTX in saliva was checked at room temperature, and at −20°C over 3 h and 3 weeks, respectively. All analyses were performed in duplicate using the above two concentrations.

RESULTS

Under the assay conditions applied, MTX and the IS were observed at 5.3 and 8.8 min, respectively (Fig. 1A). Another peak was observed constantly at the area between the two peaks at about 6.7 min. The peak was seen in the plasma samples of patients and experimental rabbits treated with MTX (Fig. 1B). The height of this peak increased linearly ($r = 0.940$) with time in a group of rabbits followed for 120 min post MTX infusion. Because it was not seen in the blanks and there was no interference by the drugs the patients were taking simultaneously with MTX, the peak is considered a metabolite of MTX. To cover a wide range of MTX concentrations, the standard curves were linear ($r = 0.999$) over two concentration ranges, 0.01–1.0 and 1.0–1000 μg/ml. The coeffecient of variation for the repeated MTX measurements was <5%. The detection limit with 100 μl injection volume was 0.01 μg/ml for MTX. The recoveries for saliva and CSF samples were higher, about 98 and 95%, compared with 71% for plasma samples (Fig. 1A).

The correlation coefficient between MTX concentration in the 50 samples measured by the two

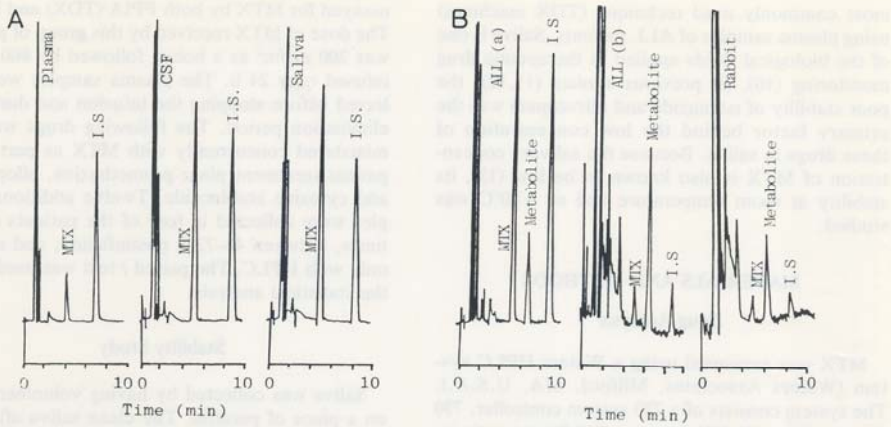

FIG. 1. Chromatograms of human, plasma, CSF, and saliva spiked with equal amounts of MTX and IS to obtain equal concentrations (A) and of ALL patient plasma samples during infusion (a) and elimination (b) and rabbit plasma during elimination (B).

methods was 0.979 (Fig. 2). The results were divided into two groups. Group A included the samples collected during infusion, and group B represents those collected during elimination (Table 1). MTX concentrations with HPLC and FPIA, (mean ± SD) were 4.62 ± 1.57 and 4.08 ± 1.64 μg/ml in group A, and 0.087 ± 0.05 and 0.112 ± 0.046 μg/ml in group B, respectively. The difference between the two methods was statistically significant, $p \geq 0.0001$ (Table 1). It was found that the ratio (peak height) of the metabolite to MTX was not the same in the two groups. The ratio was between 0.15 and 1.01 for the samples in group A and between 1.5 and 10.8 for those in group B. The HPLC assay method was also valid in tracing MTX concentrations in the plasma of four patients followed for up to 72 h postinfusion. The concentrations in these samples had at the end declined to the range of 0.008–0.022 μg/ml, which is below the minimum toxic concentration. The stability of MTX in human saliva was checked at room temperature and at −20°C over 3 h and 3 weeks, respectively. The concentrations

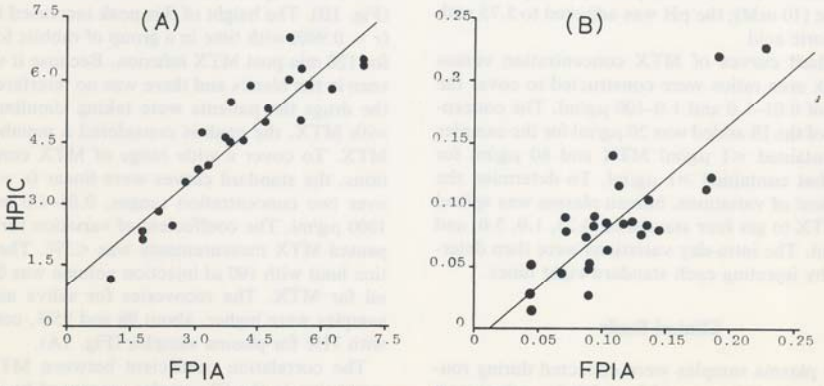

FIG. 2. The correlation between plasma MTX concentration measured by HPLC and FPIA. Patient samples collected during infusion (A), and those collected during elimination (B). In part A, $r = 0.906$; in part B, $r = 0.823$.

TABLE 1. *MTX concentrations in two groups of samples (A, B) analyzed with HPLC and FPIA and the corresponding statistical analysis*

Parameter	Group A (24-h samples, n = 24)	Group B (Elimination samples, n = 26)
HPLC (μg/ml)	4.62 ± 1.57	0.087 ± 0.05
FPIA (μg/ml)	4.08 ± 1.64	0.112 ± 0.046
Statistical analysis		
Correlation	0.906	0.823
Difference	13%	29%
p value	<0.0001	<0.0001
Metabolite/MTX		
Range	0.15–1.01	1.5–10.8
Mean ± SD	0.33 ± 0.21	3.4 ± 2.02

The ratio of the metabolite to MTX (peak height) is also reported for each group of samples.

measured were between 88 and 104% of the original concentrations prepared.

DISCUSSION

Because the time required to turn around the results of MTX is short, the assay should be simple and rapid. Few HPLC methods of MTX analysis have been published over the past 10 years (4–9). In some of these methods, the samples were directly injected onto the chromatographic system (4,5). Although the time of analysis was reduced by this procedure, the efficiency of the columns was reduced and the cost simultaneously increased. The remaining methods involved either extraction, double precipitation, or gradients elution, which required additional time and personnel involvement (6–9). The main advantage of the present method is that only a simple one-step deproteinization is required prior to chromatographic analysis. This has reduced the time of analysis to <30 min. Other advantages that may be seen in some of the previous assay methods were also maintained. For example, sensitivity is high, up to 0.01 μg/ml, which is good for tracing the level down to the nontoxic (<0.023 μg/ml) range. The result is reproducible with coefficient of variation of <5%. There was no difficulty in reconditioning the assay, which makes it suitable to run infrequent MTX samples for therapeutic drug monitoring.

The recovery of MTX in saliva and CSF is higher than that in plasma, which may be due to the lower protein contents. This advantage will be useful when analyzing MTX level in saliva and CSF, where the concentration is low and the volume collected may be small.

Currently FPIA is the method of choice for therapeutic drug monitoring (2,3). The specificity of this HPLC method was compared with FPIA on plasma samples collected from ALL patients. The correlation was linear (r = 0.979) over the concentration range of 0.014–7.0 μg/ml. HPLC tends to measure less concentration for samples collected during the elimination phase, and the reverse is true for the 24-h samples. The bias was statistically significant ($p \geq 0.0001$), particulary for the samples collected during elimination, where the difference is about 29%. Several studies have compared HPLC with either of the immunoassay methods, such as FPIA, enzyme immunoassay (EMIT), or radioimmunoassay (RIA) (14–16). They concluded that HPLC is more specific for MTX analysis because the metabolites can be separated during analysis. Crom et al. (14) pointed to the role of 7OH-MTX in this bias, particularly in the samples collected at later times during elimination. The only metabolites detected in the plasma of patients after MTX administration (17,18) were 7OH-MTX and 2,4-diamino-N^{10}-methyl pteroic acid (DAMPA). Unfortunatly, it was not possible to obtain authentic samples of these metabolites. Thus, we were unable to specify precisely the name of the metabolite, and the overestimation was related to a metabolite of MTX. In practice, MTX is usually followed until it drops below the minimum toxic concentration. The advantages we may get by using HPLC are that the concentration measured is that of MTX and the nontoxic range will be reported at an earlier time than FPIA.

The levels of most drugs in saliva correspond closely to the plasma free fraction (10). MTX is one of the few drugs that appear in saliva in a concentration that is less than the plasma free fraction (11–13). Unlike these drugs, which appear to be unstable in saliva (11,12), MTX was stable as indicated by the small change (<12%) from the original concentration.

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REFERENCES

1. Chabner BA, Donehower R, Schilsky RL. Clinical pharmacology of methotrexate. *Cancer Treat Rep* 1981;65(Suppl 1):51–4.

2. Evans WE, Crom WR, Yalowich JC. Methotrexate. In: Evans WE, Schentag JJ, Jusko WJ, eds. *Applied pharmacokinetic: principles of therapeutic drug monitoring*. Spokane, WA: Applied Therapeutics Inc. 1986:1009–56.
3. Moore MJ, Erlichman C. Therapeutic drug monitoring in oncology. Problems and potential in antineoplastic therapy. *Clin Pharmacokinet* 1987;13:205–27.
4. Breithaupt H, Kuenzlen E, Goebel G. Rapid high-pressure liquid chromatographic determination of methotrexate and its metabolites 7-hydroxymethotrexate in biological fluids. *Ann Biochem* 1982;121:103–13.
5. Tong WP, Wisnicki JL, Horton J, Ludlum DB. A direct analysis of methotrexate, dichloromethotrexate and their 7-hydroxy metabolites in plasma by high pressure liquid chromatography. *Clin Chim Acta* 1980;107:67–72.
6. Chen M, Chiou WL. Sensitive and rapid high performance liquid chromatographic method for the simultaneous determination of methotrexate and its metabolites in plasma, saliva and urine. *J Chromatogr* 1981;226:125–34.
7. Cairnes DA, Evans WE. High-performance liquid chromatographic assay of methtrexate, 7-OH methotrexate, 4-deoxy-4-amino N-methyl-pteroic acid and sulfamethoxazole in serum, urine and cerebrospinal fluid. *J Chromatogr* 1982;231: 103–10.
8. Stout M, Ravindranath Y, Kaufman R. High performance liquid chromatography assay for methotrexate utilising a cold acetonitrile purification and separation of plasma or cerebrospinal fluid. *J Chromatogr* 1981;342:424–30.
9. Alkaysi HN, Gharaibeh Abd M, Sheikh Salem MA. High performance liquid chromatographic determination of methotrexate in plasma. *Ther Drug Monit* 1990;12:191–4.
10. Horning MG, Brown L, Nowlin J, Lertratanangkoon K, Kellaway P, Zion TE. Use of saliva in therapeutic drug montoring. *Clin Chem* 1977;23:157–64.
11. Hutchings A, Spragg BP, Routeledge PA. Stability of isoniazid and acetyl isoniasid in saliva. *Ther Drug Monit* 1988; 10:234–6.
12. Hart BJ, Wilting J, DeGier JJ. The stability of benzodiazipines in saliva. *Methods Find Exp Clin Pharmacol* 1988;10: 21–6.
13. Steel WH, Stuart JFB, Whiting B, et al. Serum, tear and salivary concentrations of methotrexate in man. *Br J Clin Pharmacol* 1979;7:207–11.
14. Crom WR, Melton ER, Dodge RK, Evans WE. Evaluation of flourescence polarisation immunoassay and enzyme immunoassay of plasma methtrexate as compared with liquid chromatography [Abstract]. *Drug Intell Clin Pharm* 1984;18: 512.
15. Howel SK, Wang Y, Hosoya R, Sutow W. Plasma methotrexate as determined by liquid chromatography, enzyme inhibition assay and radioimmunoassay after high-dose infusion. *Clin Chem* 1980;26:734–7.
16. Slordal L, Prytz PS, Ingrid P, Aarbakke J. Methotrexate measurements in plasma: Comparison of enzyme multiple immunoassay technique, TD_X fluorescence polarisation immunoassay and high pressure liquid chromatography. *Ther Drug Monit* 1986;8:368–72.
17. Wang WM, Howell SK, Smith RG. Effect of metabolism on pharmacokinetic and toxicity of high dose methotrexate therapy in children [Abstract]. *Proc Amer Soc Clin Oncol* 1979;20:334.
18. Donehower RC, Hande KR, Drake JC, Chabner BA. Presence of 2,4-diamino-N^{10}-methylpteroic acid after high dose of methotrexate. *Clin Pharmacol Ther* 1979;26:63–72.