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SENSITIVE AND RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF METHOTREXATE AND ITS METABOLITES IN PLASMA, SALIVA AND URINE

MEI-LING CHEN and WIN L. CHIOU*

Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612 (U.S.A.)

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SUMMARY

A simple and sensitive high-performance liquid chromatographic assay of methotrexate (MTX) and its two active metabolites, 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N^{10}-methylpteroic acid (APA) in plasma, saliva and urine was developed. The method involved deproteinization with acetonitrile followed by addition of isoamyl alcohol and ethyl acetate. After extraction the sample was chromatographed on a cation-exchange column and monitored at 313 nm. The retention times were 5, 7 and 9 min and detection limits 20, 10 and 5 ng/ml for 7-OH-MTX, MTX and APA, respectively. For concentrations greater than 100 ng/ml one-step deproteinization of 0.1 ml sample with 0.25 ml acetonitrile was satisfactory for sample preparation. The method has been evaluated in samples from patients and rabbits receiving MTX.

INTRODUCTION

Methotrexate (MTX; 4-amino-N^{10}-methylpteroylglutamic acid), a potent antifolate, has been widely used for the treatment of various malignant diseases as well as non-neoplastic disorders [1]. With the advent of high-dose therapy followed by leucovorin rescue, plasma monitoring of MTX levels has been regarded as being mandatory to allow early detection of patients at high risk of toxicity [1—4].

Many assay methods have been developed. They include, for example, fluorometry [5], competitive protein binding [6, 7], enzyme inhibition assay [8], radioimmunoassay [9], radioassay [10], enzyme immunoassay [11] and high-performance liquid chromatography (HPLC) [12—21]. It has been reported that many non-HPLC methods lack specificity due to the potential interference of active metabolites, such as 7-hydroxymethotrexate

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(7-OH-MTX) and 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA). Furthermore, the non-HPLC methods are not capable of quantitating these two metabolites [11, 14, 20]. A major drawback of many previous pharmacokinetic studies is the use of these non-specific assay methods for the determination of MTX concentrations in biological fluids. A need for the re-evaluation of the past pharmacokinetic studies has been recently advocated [20].

In reviewing published HPLC methods, it appears that they have one or more of the following limitations or drawbacks. For example, 1 ml [12, 14, 16–18] to 3 ml [15, 21] of plasma or serum samples are needed. The sample preparations involving extraction, evaporation and reconstitution are relatively complex and may take more than 20 min [14, 16, 17]. Applicability for the determination of 7-OH-MTX [12] and APA [14–16, 19] in plasma was not studied. Retention times up to 20 or 30 min together with relatively low sensitivity for APA were reported [17, 18, 21]. In addition, only a few HPLC methods reported their application for urine analysis [13, 18, 21]. Furthermore, none of the published methods have shown their feasibility for saliva analysis. The purpose of this paper is to describe a simple, sensitive and micro HPLC assay for the simultaneous determination of MTX, 7-OH-MTX and APA in plasma, saliva and urine.

EXPERIMENTAL

Reagents and standards

All reagents were of analytical grade. MTX and APA were kindly supplied by Dr. Ven L. Narayanan from the National Institute of Health (Bethesda, MD, U.S.A.), and Dr. Maharaj K. Raina from the Lederle Labs. (Pearl River, NY, U.S.A.). The purified 7-OH-MTX was generously supplied by Dr. David Johns from the National Cancer Institute, and Dr. Kenneth K. Chan from the University of Southern California (Los Angeles, CA, U.S.A.). Additional samples used for routine standard curve study were isolated from rabbit liver homogenates on a DEAE-cellulose column according to the procedure of Watson et al. [14]. Ammonium phosphate, phosphoric acid and glass-distilled acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Isoamyl alcohol was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethyl acetate was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Most drugs tested for potential interferences of the assay were donated by the Hospital Pharmacy, University of Illinois Medical Center (Chicago, IL, U.S.A.).

Standard solutions (1 μg/ml to 10 mg/ml) of MTX and APA were prepared in distilled water. 7-OH-MTX purified from DEAE-cellulose column and dissolved in 10 m*M* Tris–HCl buffer (pH 7.5) was used for spiking directly. Its concentration was determined by comparing the HPLC peak height with those from authentic samples. All standard solutions were stored at 4°C in a refrigerator.

Sample preparations

Plasma, serum or saliva (0.2 ml) from normal subjects or patients was pipetted into 13 × 100 mm screw-capped culture tubes. The deproteinization was

carried out by adding 0.5 ml of acetonitrile, followed by vortexing for 10 sec and centrifugation at 800 *g* for 2 min. The entire supernatant was poured into a 5-ml glass tube which had a tapered base. After addition of 100 μl of isoamyl alcohol and 1 ml of ethyl acetate, the tube was vortexed for 10 sec and then centrifuged at 800 *g* for 4 min. The lower aqueous portion, 10—30 μl, was directly collected into the syringe and injected onto the column.

Urine samples were prepared by the same deproteinization procedure as described above. Since concentrations in urine are usually much higher, the deproteinized supernatant (20—50 μl) was injected directly onto the column. Peak height measurements with the assistance of a micrometer (Vernier Caliper from Fisher Scientific, Chicago, IL, U.S.A.) were used for quantitation [22]. Standard curves were constructed by supplementing blank human plasma, saliva and urine with known concentrations of MTX, 7-OH-MTX and APA.

HPLC instrumentation

The liquid chromatographic system consisted of a solvent delivery pump (Model M6000A), a fixed-wavelength detector with 313-nm filter (Model 440) obtained from Waters Assoc. (Milford, MA, U.S.A.), a syringe loading sample injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) and an ion-exchange column (Partisil PXS 10/25 SCX, 25 cm × 4.6 mm I.D., particle size 10 μm, available from Whatman, Clifton, NJ, U.S.A.). The output from the detector was connected to a 10-mV potentiometric 25.4-cm recorder (Linear Instruments, Irvine, CA, U.S.A.).

The mobile phase was prepared by mixing 10 parts of acetonitrile with 90 parts of 0.02 *M* monobasic ammonium phosphate solution acidified with phosphoric acid (0.2%). This was pumped through the HPLC system at a flow-rate of 2 ml/min, and the resulting pressure was approximately 136 bar. The recorder chart speed was 10 cm/h [23]. All experiments were carried out at ambient temperature. The optimal mobile phase used may vary with the column.

Reproducibility study

Reproducibility studies were carried out at two concentrations for each of MTX, 7-OH-MTX and APA. Six replicate analyses of plasma samples spiked with stock solutions of the three compounds to give final concentrations of 0.1 and 10 μg/ml were carried out as described earlier.

Drug interference study

Many anticancer drugs and therapy-related compounds were tested to determine if they would interfere: 5-fluorouracil, 6-mercaptopurine, adriamycin, bleomycin sulfate, cisplatin, cyclophosphamide, vincristine, vinblastine, carmustine, folic acid, folinic acid (leucovorin), 5-methyltetrahydrofolic acid, acetazolamide, hydralazine and trimethoprim. Aliquots of stock solutions of each compound were injected directly onto the column and monitored at 313 nm.

RESULTS AND DISCUSSION

Chromatograms from blank human plasma, saliva, urine and those spiked with known concentrations of MTX, 7-OH-MTX and APA, together with plasma from a patient on MTX therapy are shown in Figs. 1 and 2. The peak shape from MTX, 7-OH-MTX or APA was all symmetrical with no interferences from endogenous substances. Although there was an endogenous peak between 7-OH-MTX and MTX, it did not affect the present assay. The retention times for MTX, 7-OH-MTX and APA were 7, 5 and 9 min, respectively.

The uniqueness of this method is that a considerably shorter retention of APA (less than 10 min) was accomplished by the use of the cation-exchange column. In all the previous assays, reversed-phase [12, 13, 15—21] or anion-exchange [13—15] columns were used. It appears that reversed-phase columns could not elute APA in a short period of time presumably due to the marked difference in polarity between MTX and APA. The apparent drawback of the anion-exchange column is that a higher pH (about 7.0) of the mobile phase [13, 14] is required, which tends to deteriorate the column more rapidly.

A higher sensitivity was obtained with salivary samples than plasma as shown in Fig. 2. This might be in part due to the lower content of electrolytes in saliva which had resulted in the reduction of the final aqueous volume after the extraction. Standard curves were linear over the concentration range

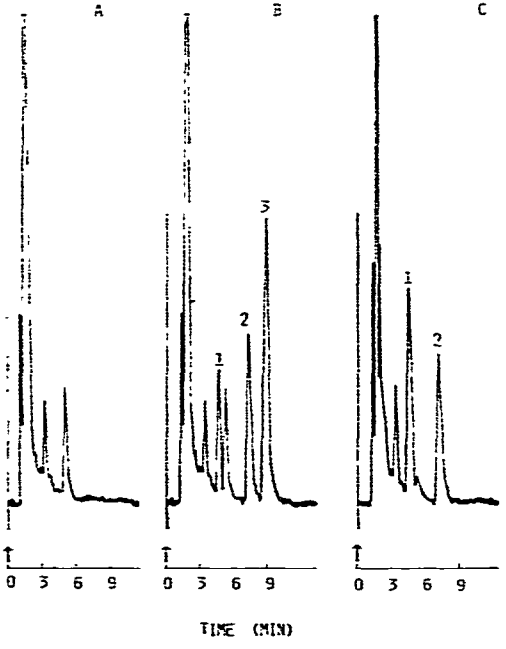

Fig. 1. Chromatograms of extracts from (A) blank human plasma; (B) plasma spiked with 0.5 μg/ml of MTX, 7-OH-MTX and APA; (C) patient plasma collected at 8 h after the end of intravenous infusion for 25 h on a dose of 750 mg/m² MTX. Peaks: 1 = 7-OH-MTX, 2 = MTX and 3 = APA. The arrow marks the point of injection. Detector sensitivity was 0.005 a.u.f.s. and recorder chart speed was 20 cm/h.

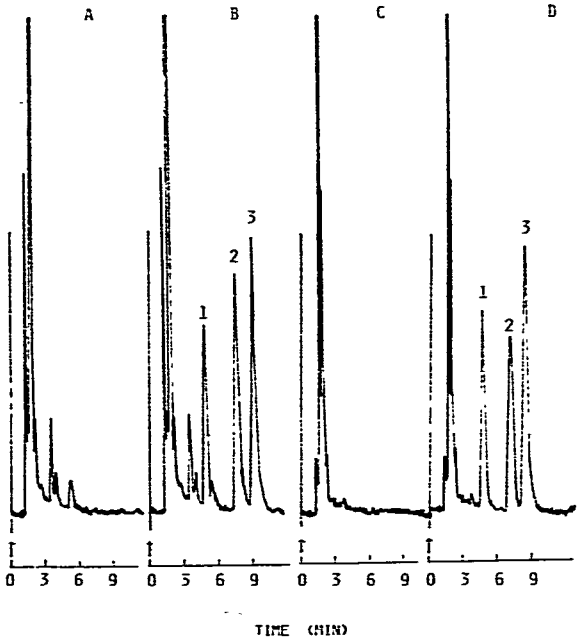

Fig. 2. Chromatograms from (A) blank human saliva extract; (B) extract of saliva spiked with 0.5 μg/ml of MTX, 7-OH-MTX and APA; (C) deproteinized blank human urine; (D) deproteinized human urine spiked with 5 μg/ml of MTX, 7-OH-MTX or APA. Peaks: 1 = 7-OH-MTX, 2 = MTX and 3 = APA. The arrow marks the point of injection. Detector sensitivity setting was 0.005 a.u.f.s. and recorder chart speed was 20 cm/h.

(0.1—10 μg/ml) studied for the three compounds (Tables I—III) as indicated by the constancy of the response factors (peak height divided by concentration).

The detection limits with a 20-μl injection volume for MTX, 7-OH-MTX

TABLE I

RESPONSE FACTORS FOR MTX, 7-OH-MTX AND APA IN HUMAN PLASMA

Spiked plasma concentration (μg/ml)	Response factor (cm/μg/ml)*		
	MTX	7-OH-MTX	APA
0.1	11.35	8.48	18.13
0.5	11.28	8.17	18.88
1	11.04	8.53	18.20
5	10.84	8.94	18.30
10	11.25	9.17	18.16
Mean ± S.D.**	11.15 ± 0.209	8.65 ± 0.396	18.33 ± 0.312
C.V.*** (%)	1.88	4.58	1.70

*Response factor = peak height/concentration; peak heights (cm) were based on the 20-μl injection and normalized sensitivity setting of 0.005 a.u.f.s.
**S.D. = standard deviation.
***C.V. = coefficient of variation.

TABLE II

RESPONSE FACTORS FROM MTX, 7-OH-MTX AND APA IN SALIVA

Spiked saliva concentration (μg/ml)	Response factor (cm/μg/ml)		
	MTX	7-OH-MTX	APA
0.1	15.35	11.30	17.25
0.5	15.22	11.32	17.12
1	15.60	11.65	17.22
5	15.72	11.45	17.44
10	16.07	11.58	17.66
Mean ± S.D.	15.59 ± 0.332	11.46 ± 0.155	17.33 ± 0.214
C.V. (%)	2.13	1.35	1.23

and APA in plasma are 15, 25 and 10 ng/ml, respectively. Higher sensitivity could be obtained when 0.5 ml rather than 1 ml of ethyl acetate was used for extraction. This was primarily attributed to the lower volume of the final aqueous solution obtained after extraction. The above modification could result in detection limits down to 10 ng/ml (approximately $2 \cdot 10^{-8}$ *M*) for MTX, 20 ng/ml for 7-OH-MTX and 5 ng/ml for APA.

The intra-day coefficients of variation for three compounds were between 1.23 and 5.28%. Under the condition described, three replicate analyses in three days gave inter-day coefficients of variation of 4.6% for MTX, 7.9% for 7-OH-MTX and 3.8% for APA.

Although there is no internal standard used in the present assay, excellent reproducibility was obtained as shown in Table IV. The extraction efficiencies for plasma samples were 70, 50 and 77% for MTX, 7-OH-MTX and APA, respectively. Recoveries for saliva samples were higher, being 98, 61 and 79% for these three compounds, respectively. Compared with many other HPLC methods, the present assay offers higher recovery, lower limit of sensitivity as well as lower coefficients of variation for MTX and its two metabolites in plasma [14—18, 20].

TABLE III

RESPONSE FACTORS FOR MTX, 7-OH-MTX AND APA IN URINE

Spiked urine concentration (μg/ml)	Response factor (cm/μg/ml)		
	MTX	7-OH-MTX	APA
1	1.20	1.0	1.75
2.5	1.07	0.98	1.77
5	1.04	1.09	1.71
7.5	1.09	1.11	1.68
10	1.10	1.07	1.73
Mean ± S.D.	1.10 ± 0.058	1.05 ± 0.055	1.72 ± 0.034
C.V. (%)	5.28	5.23	1.94

TABLE IV

REPRODUCIBILITY DATA FOR MTX, 7-OH-MTX AND APA IN HUMAN PLASMA

Compound	0.1 μg/ml (n = 6)			10 μg/ml (n = 6)		
	Mean peak height* (cm)	S.D.	C.V. (%)	Mean peak height** (cm)	S.D.	C.V. (%)
MTX	1.135	0.079	7.0	5.625	0.150	2.67
7-OH-MTX	0.848	0.038	4.4	4.587	0.103	2.25
APA	1.813	0.136	7.5	9.082	0.353	3.89

*Based on 20-μl injection at a sensitivity setting of 0.005 a.u.f.s.
**Based on 20-μl injection at a sensitivity setting of 0.1 a.u.f.s.

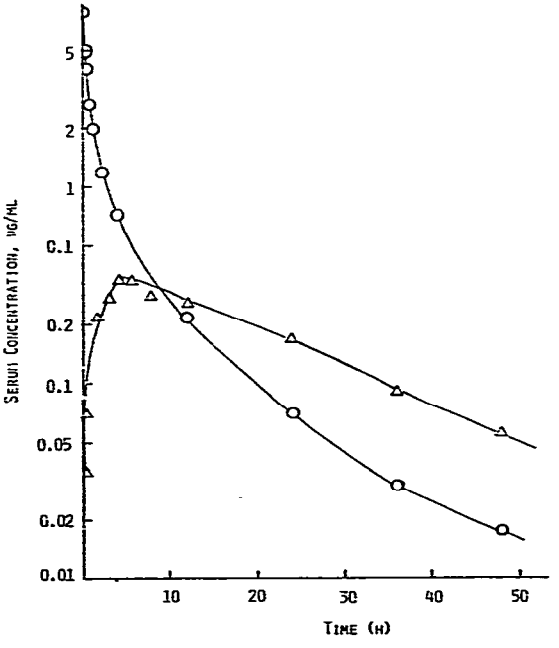

Fig. 3. Serum concentration profiles of MTX (o——o) and 7-OH-MTX (△——△) in a patient following intravenous administration of 40 mg/m² of MTX.

The results of interference studies showed that none of the drugs tested interfered with the analysis of MTX and its two metabolites.

By using the simple deproteinization method, MTX concentrations in urine can be readily detected down to 0.1 μg/ml (about $2 \cdot 10^{-7}$ M). This method is also applicable to plasma with MTX levels above 0.1 μg/ml. Therefore, analysis of drug levels in plasma or serum by the one-step deproteinization procedure might be adequate for routine monitoring in high-dose therapy [1].

Typical plasma level profiles from a patient receiving 40 mg/m² of MTX by intravenous push and from a rabbit after 15 mg/kg intravenous bolus injection are shown in Figs. 3 and 4, respectively. No APA was found in the patient's plasma, saliva and urine collected up to 72 h. This was probably due to the much lower concentrations of APA present in biological fluids or to

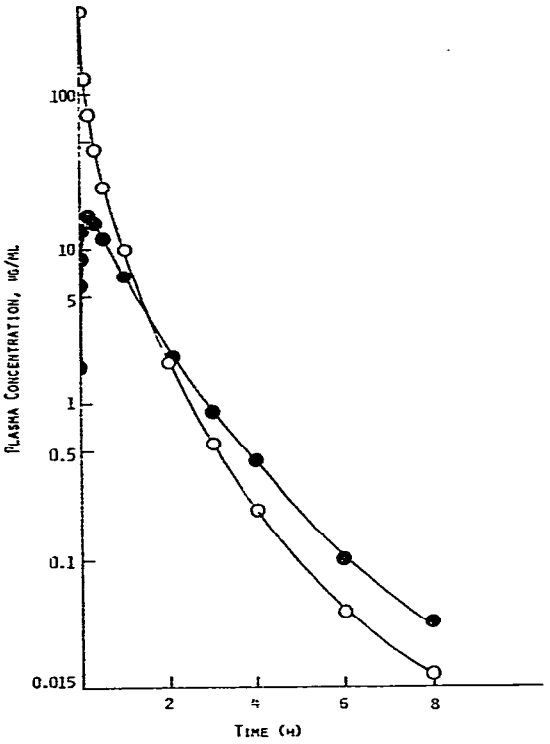

Fig. 4. Plasma concentration profiles of MTX (o——o) and 7-OH-MTX (•——•) from a rabbit following intravenous administration of 15 mg/kg of MTX.

the failure of the production of APA in this patient. In this case, APA can be used as an internal standard for the assay. Our data indicate that MTX is eliminated from the rabbit by multi-exponential decay with the terminal half-life much longer than 25 min which was reported previously [18].

Acetonitrile appears to be an ideal deproteinizing agent. The volume ratio of 2.5 between acetonitrile and plasma, saliva or urine was satisfactory to assure virtual completeness of the deproteinization process. Such a simple deproteinization method has been successfully used in the assay of creatinine [24], gentamicin [25], procainamide [26], tolbutamide [27], furosemide [28], and other drugs developed from this laboratory.

In attempting to increase the sensitivity of the assay, efforts have been made by acidification or alkalization during the extraction procedure. Surprisingly, the peak heights were all decreased, indicating the reduced extraction efficiency in both cases. Addition of isoamyl alcohol in the present assay was found to enhance the sensitivity by 1.5- to 2-fold for the three compounds. Due to the high viscosity of this reagent, the use of a syringe (Hamilton, Reno, NV, U.S.A.) rather than a micro pipettor is suggested to assure complete delivery. The final volume of aqueous phase would be constant if an accurate amount of isoamyl alcohol was introduced. Careful sample preparation prior to injection onto the column is essential in reducing analytical errors. The use of a micrometer also increases the accuracy of the measurement of peak heights.

The method described here permits a rapid, simultaneous determination of MTX and its two active metabolites in biological fluids. The sample preparation prior to chromatography is easy and no evaporation or reconstitution steps are needed. In view of the simplicity, specificity and sensitivity, the method may be of great use in pharmacokinetic studies in humans and animals. It may also be suitable for routine monitoring of serum levels of MTX as well as its two metabolites, 7-OH-MTX and APA, which have received wide attention recently due to their implication in nephrotoxicity during MTX therapy [1, 29—31].

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