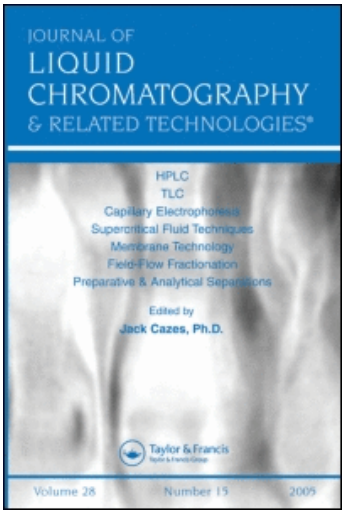

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Determination of Trace Methotrexate and 7-OH-Methotrexate in Plasma by High-Performance Liquid Chromatog-Raphy with Fluorimetric Detection

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DETERMINATION OF TRACE METHOTREXATE AND 7-OH-METHOTREXATE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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ABSTRACT

A sensitive and reliable HPLC method consisting of a solid-phase extraction, post-column photoreaction of the analytes in a polyethylene tubing by UV lamp, and fluorimetric detection for the simultaneous determination of methotrexate (MTX) and 7-OH-methotrexate (7-OH-MTX) in plasma was developed. The linear relationships between the peak area and MTX (0.1 ng/ml-1000 ng/ml, r=0.9997) or 7-OH-MTX (6.25 ng/ml-400 ng/ml, r=0.9995) concentrations were obtained. The average coefficients of variation for the intraday and interday replications were less than 11% and the absolute recoveries were 96.8%-101.2% for MTX and 76.4%-86.7% for 7-OH-MTX for the calibration ranges used. The limit of detection for MTX in plasma was 0.05 ng/ml. This most sensitive HPLC method reported so far was used for the trace assay of plasma MTX concentrations in dogs following a topical application of MTX in gel.

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INTRODUCTION

Methotrexate(MTX), an antifolate used for the treatment of several types of cancer, has frequently been prescribed in the therapy of rheumatoid arthritis. Although the complete mechanism of MTX for the treatment of rheumatoid arthritis is yet to be known, the actions of antiinflammation and immunosuppression of this drug have been found [1, 2]. The recommended dose of MTX for the patients with rheumatoid arthritis is 10 - 20 mg per week by oral, intravenous or intramuscular administration. In order to maintain an effective therapeutic concentration of MTX in plasma for a longer duration and reduce the side effects due to a dose dumping, a transdermal formulation of MTX is being developed in our laboratory. Since the drug concentrations in plasma following transdermal application were expected to be much lower than that following oral administration due to the barrier effect of the stratum corneum, a sensitive analytical method which allows the quantitation of the drug concentration in plasma down to 10^{-11} g/ml was needed to evaluate the bioavailability and pharmacokinetic profiles of the transdermal formulation in the body. Various methods have described the determination of MTX in biological fluids which include an enzyme multiplied immunoassay [3], fluorescence polarization immunoassay [4], radioimmunoassay [5,6], capillary zone electrophoresis

[7] and HPLC [8-20]. In terms of high degree of specificity and sensitivity, HPLC provides a powerful technique for monitoring the drug concentration in biological samples. HPLC methods with UV [8-10], fluorescence [11-13] and electrochemical detection [14-16] have been reported for this compound, and various procedures for sample treatment such as protein precipitation with TCA or PCA [17], liquid extraction [18] and solid extraction [18-20] were used. Salamoun et al. [12] first reported the use of a PTFE capillary tubing for the post-column photodecomposition of MTX. Nuernberg et al.[19] described a HPLC method using a solid-phase extraction procedure and UV detection for MTX and its metabolites in biological samples. Another recently developed HPLC method using solid-phase extraction and a similar post-column photoreaction procedure with fluorescence measurement offered the detection limit down to 0.095 ng/ml [20]. Based on the work of these previous authors [12,19,20], we were able to improve the method with respect to the sensitivity and photoreaction procedures. In this paper, a very sensitive and reliable HPLC method for the quantitation of MTX and 7-OH-MTX consisting of a solid-phase extraction and fluorimetric detection following post-column photochemical degradation of the parent compounds by UV irradiation in a polyethylene tubing is presented.

EXPERIMENTAL

Chemicals

MTX (obtained from American Cyanamid Company, Pearl River, NY) and 7-OH-MTX (kindly provided by Dr. David Johns of the National Cancer Institute, Rockville, MD) were used as received. HPLC-grade acetonitrile and methanol and all the other analytical-grade chemicals used were purchased from commercial sources. The water was treated with a Millipore water purification system. Bond-Elut cartridges (100 mg) were obtained from Varian Analytical Supplies Company (Harbor City, CA).

Solid-phase extraction

Each Bond-Elut cartridge was conditioned by washing with 1 ml of methanol first and with 1.5 ml of phosphate buffer (0.05 M, pH 2.7) before the application of sample. One ml of plasma (spiked or dosed) samples was mixed with an equal volume of a phosphate buffer (0.05 M, pH 6.5), and applied to the cartridge. The flow rate was maintained approximately at 2 ml/min by a mild vacuum. The cartridge was washed sequentially with 2 ml of phosphate buffer (0.05 M, pH 2.7), 1 ml of sodium hydroxide (0.1 M) and 1 ml of the phosphate buffer. The adsorbed compounds were eluted with 1.5 ml of methanol. The eluate was evaporated to dryness under nitrogen at an ambient temperature. The residue was dissolved with 0.2 ml of the mobile phase and 0.1 ml of the aliquot was injected onto the column.

Apparatus

An Altex Model 110A pump, equipped with a Rheodyne 7125 injector and a reversed-phase column (Novapak C_{18}, 5μm, 150 × 3.9 mm I.D., Phenomenex, Rancho Palos Verdes, CA), a guard column (C_{18}, spherisorb, 30 μm, 30 x 3.2 mm I.D.), a Spectroline pencil UV lamp (254 nm) inserted in a polyethylene tubing coil (PE-20, I.D. 0.38 mm, O.D. 1.09 mm, length 3 m) which was connected between the column and the monitor for the photodecomposition of MTX and 7-OH-MTX, a Shimadzu RF-530 fluorimetric monitor and a Shimadzu C-R 3A integrator (Shimadzu Co. Tokyo, Japan) were used.

Chromatographic conditions

The mobile phase consisted of a 0.014 M of phosphate buffer (pH 6.5) containing 4% of N,N-dimethylformamide, 3.3% of acetonitrile and 0.5% of 3% hydrogen peroxide. The operation was conducted at ambient temperature and the flow rate was 1 ml/min. The fluorescence excitation and emission wavelengths were set at 350 nm and 465 nm, respectively.

Quantitation

The calibration curves for MTX and 7-OH-MTX in plasma were constructed daily by spiking blank plasma samples with known amounts of the compounds. The MTX and 7-OH-MTX concentrations in the plasma samples were obtained by comparison of the peak area of the sample chromatogram with the calibration plots.

RESULTS

Chromatogram

The typical chromatographic profiles of MTX and 7-OH-MTX in human and dog plasma were shown in Fig 1. Using the solid-extraction method, clean baselines without any interfering peaks were obtained. The retention times of MTX and 7-OH-MTX were 4.0 and 5.5 min, respectively. A chromatogram of a blank human plasma sample spiked with 0.1 ng/ml of MTX was shown in Fig 1 (C).

Linearity and limit of detection

As shown in Fig 2 and Fig 3, the responses to MTX and 7-OH-MTX concentrations in the human plasma were highly linear over the range of 0.1 - 1000 ng/ml ($r=0.9997$, $n=7$) and 6.25 - 400 ng/ml ($r=0.9995$, $n=6$) for MTX and 7-OH-MTX, respectively. The similar linearity was also obtained in the dog plama. The limit of detection which is the smallest concentration that can be distinguished from the blank (the ratio of signal/baseline noise = 3) was 0.05 ng/ml of plasma for MTX and 2 ng/ml for 7-OH-MTX when 1 ml of plasma sample was used. The CV for the quantitaton of 0.1 ng/ml of MTX in plasma was 17.3% ($n=3$).

Recovery and precision

The absolute recoveries from plasma were found to be 96.8% - 101.2% for MTX over the range of 1 ng/ml to 100 ng/ml and 76.4% - 86.7% for 7-OH-MTX over the range

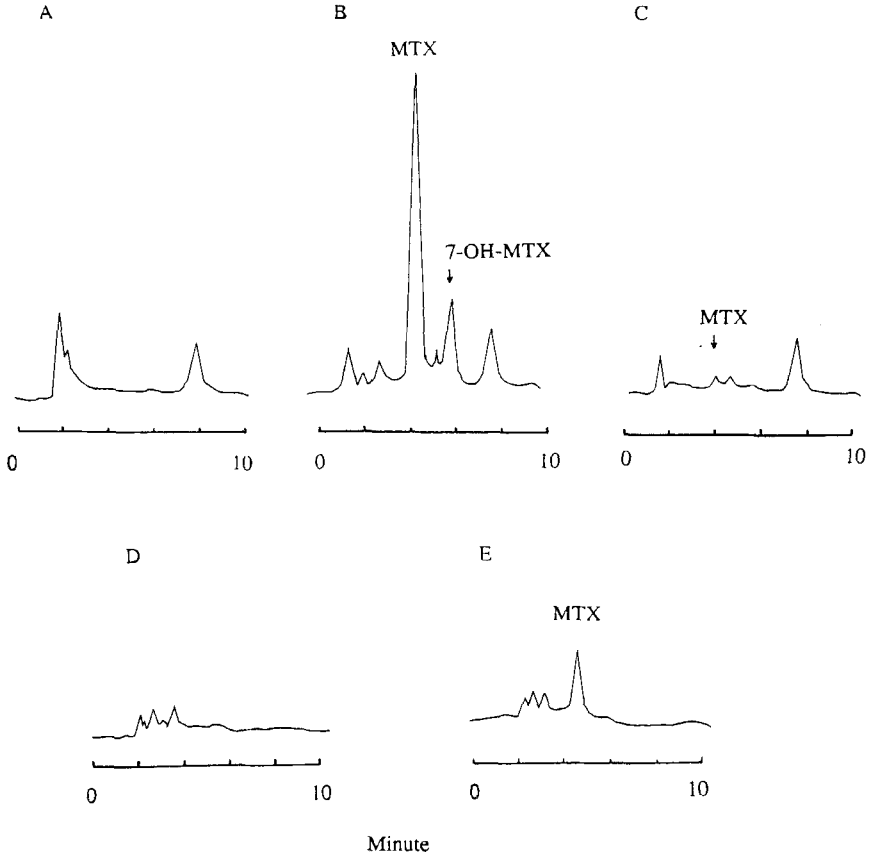

Figure 1. Chromatograms of MTX and 7-OH-MTX in human and dog plasma. A: blank human plasma; B: human plasma containing 10 ng/ml of MTX and 20 ng/ml of 7-OH-MTX; C: human plasma containing 0.1 ng/ml of MTX; D: blank dog plasma; E: dog plasma containing 1 ng/ml of MTX.

of 10 ng/ml to 100 ng/ml, as shown in Table 1.

The variation of intraday and interday assays for the determination of MTX and 7-OH-MTX were shown in Table 2. The CV's for the assays were 3.9% - 8.9% for MTX in the range of 1 ng/ml - 200 ng/ml and 5.6% - 10.8% for 7-OH-MTX in the range of 20 ng/ml - 200 ng/ml. The average

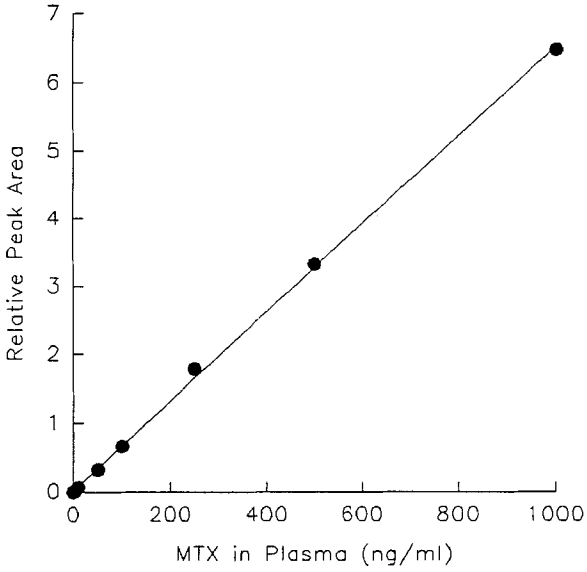

Figure 2. Standard curve for MTX in human plasma. Each point is the mean of duplicate samples. (r=0.9997)

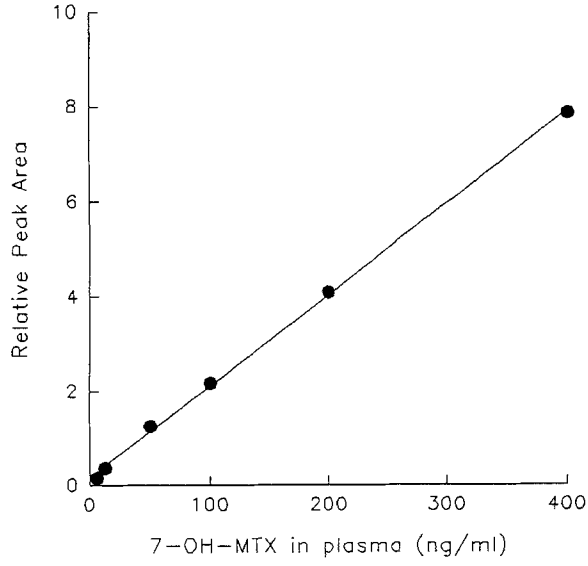

Figure 3. Standard curve for 7-OH-MTX in human plasma. Each point is the mean of duplicate samples. (r=0.9995)

TABLE 1. Recoveries of MTX and 7-OH-MTX (mean ± SD)

MTX			7-OH-MTX		
Conc. (ng/ml)	Recovery (%)	n	Conc. (ng/ml)	Recovery (%)	n
1	97.2 ± 8.1	6			
10	101.2 ± 9.1	6	20	76.4 ± 4.3	6
100	96.8 ± 3.9	6	200	86.7 ± 9.4	6

TABLE 2. Precision of the Assay for MTX and 7-OH-MTX

		conc. (ng/ml)	n	CV (%)
Intraday	MTX	1	6	8.3
		10	6	8.9
		100	6	3.9
	7-OH-MTX	20	6	5.6
		200	6	10.8
Interday	MTX	10	9	7.1
	7-OH-MTX	25	5	5.5

CVs for the determination of interday assays were 7.1% for MTX and 5.5% for 7-OH-MTX.

Effects of coil material on photoreaction

Three coils which were made with teflon (I.D. 0.88 mm, O.D. 1.75 mm, length 1 m), quartz (I.D. 1 mm, O.D. 2.5 mm, length 0.9 m) and polyethylene(I.D. 0.86 mm, O.D. 1.27 mm, length 1 m) were used to compare the efficiency of the photochemical reaction of MTX. The chromatogram with the coils made by different materials were shown in Fig 4. The coil made by polyethylene tubing was shown to

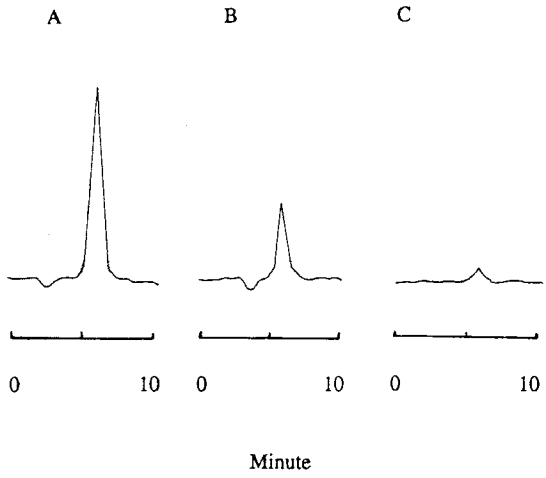

Figure 4. Chromatograms of MTX (50 ng/ml) with different coils used for the photochemical reaction. A: polyethylene tubing; B: quartz tubing; C: teflon tubing.

be the most suitable photoreaction device among the three coils tested. It was also found that the sharper chromatographic peaks for MTX and 7-OH-MTX were observed when a coil made by a thinner polyethylene tubing (PE-20 tubing, I.D. 0.38 mm, O.D. 1.09 mm) was used.

The effect of the length of PE-20 tubing on the photoreaction of these compounds was investigated. The relative peak areas (n=2) after injection of 50 μl of MTX (20 ng/ml) onto the column were 6981, 17239 and 13945 for the coils of the different length of 2 m, 3 m and 4 m, respectively. Thus the PE-20 coil with the length of 3 m was chosen for the assay of the compounds throughout this study.

Selection of the fluorescence excitation and emission maxima

The excitation wavelengths from 330 nm to 370 nm were tried for the comparison of the fluorescence intensity of the MTX products. The highest response was observed at 350 nm which was the same as the excitation wavelength used by Beck et al.[20].

The variation of the peak area of the photodegraded MTX with different emission wavelength used was shown in Fig 5. The maximum peak area was observed at 465 nm which was different from 436 nm used by Beck et al.[20].

Application

Four beagle dogs (10-14 kg) were used for the bioavalability study of a transdermal gel formulation of MTX. The hair on the two right legs were carefully shaved and the 8% of MTX gel (2.5 mg/kg) was applied on the joints (100 cm^2). The blood samples were obtained at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hr after the application and analyzed by the present method. The lowest concentration which was quantitated by this method was 1 ng/ml of MTX and one of the chromatograms was shown in Fig 1 (E). The steady state concentration of 47 ng/ml was reached at 4 hr after the dosing and maintained for about 30 hrs. The plasma profile was shown in Fig 6. The 7-OH-MTX concentration was undetectable in these plasma samples.

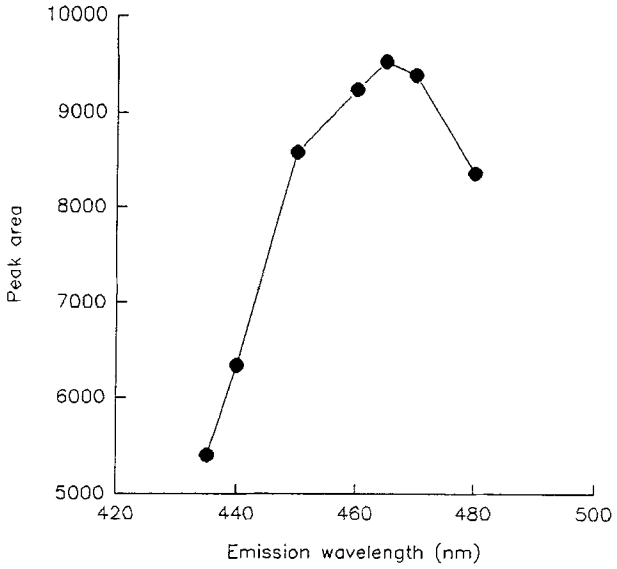

Figure 5. The effect of emission wavelength on the relative peak area of MTX (10 ng/ml).

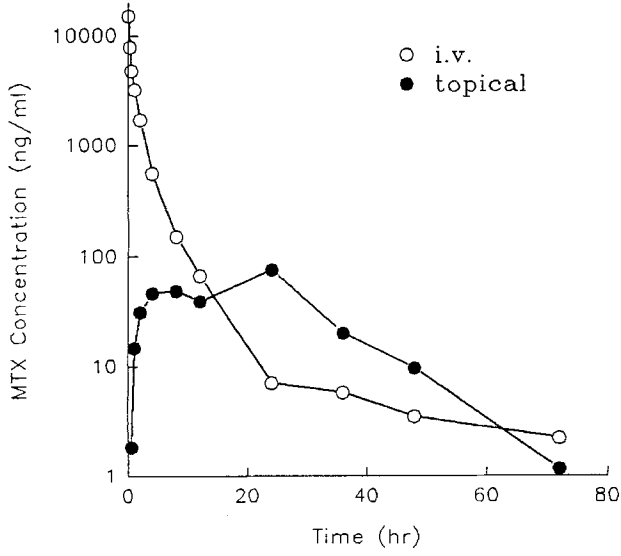

Figure 6. Plasma level versus time profiles of MTX in a dog after an i.v. injection and a topical application with 3 g of gel containing 25 MTX.

DISCUSSION

Various procedures for the sample preparation were tested. Plasma deproteinization with acetonitrile, trichloroacetic acid or perchloric acid provided a simple and fast preparation of samples, but resulted in a low recovery of the compounds and interfering peaks in the chromatogram, especially at the lower concentration range used. The solid-phase extraction method was more effective not only in terms of removing the interfering peaks but also with respect to the specificity and sensitivity of the assay. The results observed in this study were generally in agreement with those reported in the literature [17,19,20]. As compared to the solid-phase extraction reported by Nuernberg et al.[19], the current method used a smaller Bond-Elut cartridge (100 mg) which required smaller volumes of the liquids for extraction, and therefore a shorter sample preparation time. In addition to the reduced expenses for the cartridges and solvents used, excellent linear relationships between the analyte concentrations and peak areas over a wide range of concentration were obtained.

The major photodegradation product of MTX which shows an intense fluorescence was reported to be 2,4-diaminopteridine-6-carboxaldehyde [12]. The post-column photodecomposition of MTX and 7-OH-MTX to the fluorescent products was achieved by the UV irradiation of the samples in the polyethylene tubing coiled around the UV

lamp which was connected between the analytical column and the detector. The effect of the tubing materials on the photoreaction of the compounds was examined and the efficiency of photodegradation among the polyethylene, teflon and quartz tubing was shown in Fig 4. The smallest peak was observed when the teflon tubing was used which was probably due to a limited penetration of the UV light through the walls of teflon tubing. When the quartz tubing was used for the photoreaction, the fluorescence response of the MTX product was only a one-third of that of the polyethylene tubing. The high fluorescence intensity observed for the MTX degradation with the polyethylene tubing indicated that not only the UV light sufficintly penetrated through the walls of polyethylene tubing, but also effectively induced the photochemical reaction of the analytes in the coil. The efficiency of the polyethylene coil for the photoreaction appeared to remain unaffected during 250 hr of use. The peak heights for 50 μl sample of MTX (50 ng/ml) were 2.32 ± 0.2 cm for the new coil and 2.45 ± 0.18 cm for the old coil which was used for about 250 hr. Although either the polyethylene or quartz tubing could be used for the post-column photoreaction of MTX and 7-OH-MTX, the former was chosen because of its availability, low cost and easy handling.

The maximum fluorescence intensity of MTX was obtained at the excitation wavelength of 350 nm and the

emission wavelength of 465 nm [20]. At this emission wavelength, it was found that nearly 40% increase in the fluorescence response was observed as compared to the emission wavelength of 435 nm previously used. A chromatogram of a plasma sample containing 0.1 ng/ml of MTX was shown in Fig 1 (c). A small but sharp and symmetrical peak for the MTX degradation product was observed at this low concentration. Using this method we have determined the bioavailability of the MTX transdermal gel applied in dogs, which was approximately 12% of the dose applied for the first 72 hrs. Fig 6 shows the plasma level versus time profiles of MTX for the i.v. and transdermal gel formulation.

In conclusion, a very sensitive, reliable and economical HPLC method for the determination of MTX in plasma using a solid-phase extraction, post-column photoreaction of the analytes in a polyethylene tubing by UV irradiation, and fluorimetric detection was presented. Both MTX and its main metabolite, 7-OH-MTX, were quantitated simultaneously with high accuracy and precision. The method was used for the analyses of MTX in the dosed plasma samples of dogs.

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