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Sensitive determination of methotrexate in monkey plasma by high-performance liquid chromatography using on-line solid-phase extraction

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Abstract

A high-performance liquid chromatgraphic method was developed for the sensitive determination of methotrexate (MTX) in monkey plasma using direct injection and on-line solid-phase extraction. After application of a 100-μl aliquot of plasma to a pre-treatment column, the column was washed with 0.02 *M* phosphate buffer (pH 7) to eliminate plasma proteins and endogenous substances, and subsequently the adsorbed MTX was eluted. The MTX fraction was transferred to an analytical column by a column-switching (heart-cutting) technique, and MTX was analyzed using ion-pair chromatography with tetrabutylammonium bromide. More than 50 injections could be performed onto one pretreatment column. The accuracy, precision, reproducibility and linearity were satisfactory over a wide range of MTX concentrations (5–1000 ng/ml). The quantitation limit was 5 ng/ml with a signal-to-noise ratio of 5. The method was suitable for the pharmacokinetic study of MTX in monkey.

1. Introduction

Methotrexate (MTX) is an anti-metabolite widely used in the treatment of various malignant and non-malignant diseases. Low-dose MTX therapy is known to be effective in the treatment of rheumatoid arthritis with a relatively low incidence of serious toxicity [1,2]. However, a life-threatening interaction has been reported in patients receiving a non-steroidal anti-inflammatory drug (NSAID) together with MTX [3–5]. Information on the pharmacokinetics of MTX, in particular with regard to the interaction with NSAID, is of increasing importance. Although Najjiar et al. [6] reported the effect of indometachin on the pharmacokinetics of MTX in rabbits, the plasma level of MTX was too high to derive accurate information on the interaction.

Although various methods for the determination of MTX in biological materials have been published, the majority of those was established for pharmacokinetic studies in anti-cancer therapy (high-dose therapy) [7–12]. The sensitivity of these methods is insufficient for pharmacokinetic studies in rheumatoid therapy (low-dose therapy), which requires a sensitivity of less than 10 ng MTX per millilitre in plasma. Some sensitive methods have also been reported for the determination of MTX, however, these are

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based on either radioimmunoassay or laborious high-performance liquid chromatography (HPLC) procedures. Large sample volumes, solid-phase extractions or post-column photo-oxidative reactions were required in these methods [13–16].

In the present study an on-line solid-phase extraction technique folowing direct sample injection was investigated in order to develop a sensitive and simple HPLC method for the determination of MTX in plasma, and the technique was applied to the pharmacokinetic study of MTX in monkey.

2. Experimental

2.1. Materials

Biochemical grade MTX and HPLC grade acetonitrile were purchased from Wako (Osaka, Japan). Tetra-*n*-butylammonium bromide (TBAB, ion-pair chromatographic grade) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Deionized water was further purified using the Milli-Q water purification system (Nihon Millipore, Yonezawa, Japan). All other chemicals were of reagent grade and used without further purification. Drug free plasma was obtained from cynomolgus monkey. Phosphate buffer (0.02 M, pH 7) was prepared by mixing 0.02 M disodium hydrogenphosphate and 0.02 M potassium dihydrogenphosphate.

2.2. Instrumentation and HPLC conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of three LC-6A pumps, an SPD-6AV UV-Vis detector, an FCV-2AH six-port switching valve and an SIL-6B autosampler, all of which were controlled by an SCL-6B controller. UV detection was performed at 305 nm. The peak height of MTX was measured by a Waters 805 data station (Waters, Osaka, Japan). Samples injected to HPLC system were kept at 4°C by a WIG 7000A cooling system (Ishido, Chiba, Japan) before analysis.

The pretreatment column (C1) was a Guardpak μBondapak C_{18} (10-μm particle size, Cat. no. 88070; Waters, Milford, MA, USA). The analytical column (C2) was an Inertsil ODS-2 column (A type, 5-μm particle size, 150 × 4.6 mm I.D.; GL Science, Tokyo, Japan). The mobile phases (MP1, MP2 and MP3) had the following composition: MP1, 0.02 *M* phosphate buffer (pH 7); MP2, 0.02 *M* phosphate buffer (pH 7)–acetonitrile (4:6, v/v); and MP3, 0.02 *M* phosphate buffer (pH 7)–acetonitrile (82:18, v/v) containing 15 m*M* TBAB. The columns (C1 and C2) were kept at ambient temperature and the flow-rates of the mobile phases were 1 ml/min.

2.3. Analytical system and procedure

A schematic diagram of the HPLC system is shown in Fig. 1. The time program of system is described in Table 1. MTX injected was retained on C1 with MP1, while plasma proteins and endogenous substances were washed to waste. At 16 min after injection, MTX was eluted with a stepwise gradient of MP1 and MP2 (MP1–MP2; 90:10, v/v) and transferred to C2 by a heart-cut technique between 19.80 and 22.60 min. MTX introduced on C2 was eluted with MP3 and was monitored at 305 nm. C1 was washed with MP2 for about 7 min and then equilibrated with MP1 for 10 min. The sample analysis was completed within 40 min.

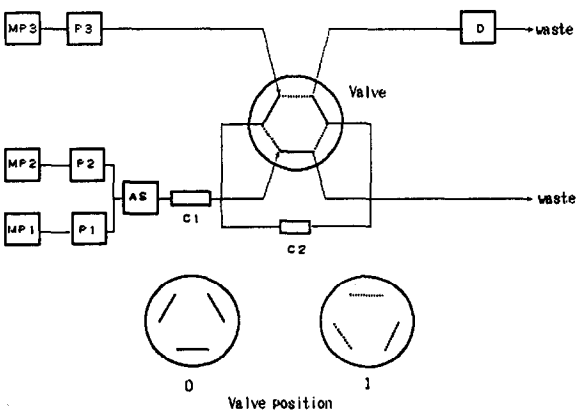

Fig. 1. Set up of the HPLC system. MP1–MP3, mobile phases 1–3; P1–P3, pumps 1–3; AS, auto sampler; C1, C2, columns 1 and 2; D, detector.

Table 1
Time program for the column-switching system

Time (min)	MP1 concentration (%)	MP2 concentration (%)	Valve position
0	100	0	0
16	100	0	
16.01	90	10	
19.80			1
22.60	90	10	0
22.61	0	100	
30.00	0	100	
30.01	100	0	
40.00		(Stop)	

2.4. Sample preparation

Monkey plasma was filtered through a 0.45-μm membrane filter and a 100-μl aliquot was injected onto the HPLC system.

3. Results

3.1. Effect of direct injection on the performance of the pre-treatment column

The effect of direct injection of monkey plasma on the performance of the pre-treatment column (C1) was investigated. Fig. 2 shows chromatograms of MTX using a new C1 column (1) and a column already used for 30 plasma injections. Only after 30 repeated injections of 100-μl aliquots of plasma peak broadening appeared.

3.2. Effect of TBAB on compression of the MTX peak in the analytical column

The effect of TBAB on compression of the MTX peak in the analytical column (C2) was investigated. Fig. 3 shows the ratio of the peak height of MTX obtained by the column switching technique to that obtained by direct injection onto C2. The acetonitrile content in MP3 was adjusted as the retention times of MTX were almost the same with and without TBAB in MP3. When a fresh C1 was used, comparable peak heights were obtained with and without column switching at a concentration of TBAB higher than 10 m*M*. In the case of a C1 column used for 30 repeated injections of 100 μl aliquots of plasma, comparable peak heights were also obtained at 15 m*M* TBAB.

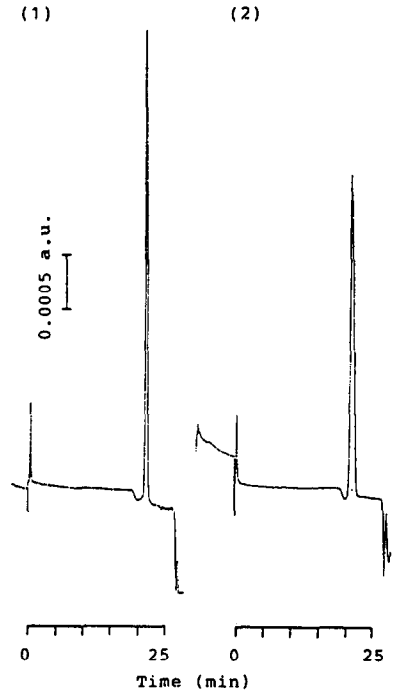

Fig. 2. Chromatograms of MTX obtained with C1 (1) before and (2) after 30 repeated injections of 100-μl aliquots of monkey plasma.

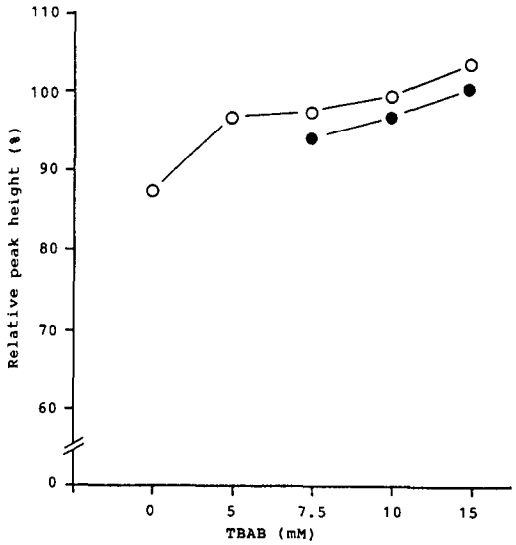

Fig. 3. Effect of TBAB concentrations on the relative peak height of MTX. Peak height was taken as 100% when MTX was directly injected onto C2. MP3 composition: 0.02 *M* phosphate buffer (pH 7)–acetonitrile (92.5:7.5, v/v) or (82:18, v/v) containing TBAB (5, 7.5, 10 and 15 m*M*). Open circles and closed circles show the peak heights for C1 before and after 30 repeated injections of 100-μl aliquots of monkey plasma (see Fig. 2), respectively.

3.3. Chromatograms and validation data

Typical chromatograms of drug-free monkey plasma and of plasma spiked with MTX are shown in Fig. 4. No peak was observed at the retention time of MTX in fresh blank plasma and in blank plasma allowed to stand for 2 days at 4°C. Analytical validation data were obtained using plasma spiked with MTX. The accuracy, precision and reproducibility were satisfactory (Table 2). A linear relationship was obtained for the range 5–1000 ng/ml (Table 3). The quantitation limit of MTX was 5 ng/ml with a signal-to-noise ratio of 5. At least 50 repeated injections could be made onto one pre-treatment column, since comparable peak heights were obtained for the 1st and 50th injection of plasma spiked with MTX. MTX spiked to monkey plasma was stable at least for 2 days at 4°C.

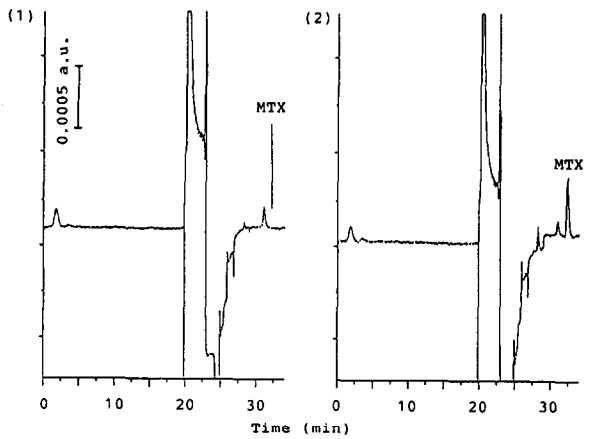

Fig. 4. Typical chromatograms of (1) drug free plasma and (2) plasma spiked with MTX (20 ng/ml).

4. Discussion

Recently, various types of restricted-access-phase columns have been developed for the direct injection analysis of biological fluids [17–20]. These restricted-access-phase columns are useful, although some problems have been pointed out, i.e. small sample volume capacity, low performance and expensiveness [19–21]. A commercially available inexpensive guard column (Guard-pak μBondapak C_{18}) has been used for on-line solid-phase extraction [22]. This article prompted the authors to use Guard-pak μBondapak C_{18} (C1) for pre-treatment and on-line solid-phase extraction. As shown in Fig. 2, peak broadening appeared by injection of the plasma. A similar loss of column performance was observed when an L-column (Chemical Inspection and Testing Institute, Tokyo, Japan), one of the restricted-access-phase columns, was used as the pre-treatment column. Thus, deterioration in the column efficiency of C1 appeared to be inevitable when direct injection was applied to routine analysis. To reduce the effect of deterioration, however, studies were performed on the HPLC conditions.

To compensate for the deterioration in the column efficiency of C1, compression of the MTX peak was attempted by increasing the acetonitrile content in the mobile phase (MP3) after column switching. Although satisfactory peak compression was obtained, MTX was hard-

Table 2
Accuracy, precision and reproducibility of the determination method for MTX in monkey plasma

Day		Nominal concentration (ng/ml)		
		5	100	1000
Intra-day assay (n = 5)				
1	Observed conc. (ng/ml)	5.13	98.41	1008.66
	R.E. (%)[a]	2.60	−1.59	0.87
	C.V. (%)	8.77	4.92	4.78
2	Observed conc. (ng/ml)	5.00	97.48	993.36
	R.E. (%)	0.00	−2.52	−0.66
	C.V. (%)	10.00	2.22	1.89
3	Observed conc. (ng/ml)	5.08	100.74	1015.74
	R.E. (%)	1.60	0.74	1.57
	C.V. (%)	12.20	1.54	1.93
Inter-day assay (n = 3)				
	Observed conc. (ng/ml)	5.07	98.88	1005.92
	R.E. (%)	1.40	1.12	0.59
	C.V. (%)	1.30	1.70	1.14

[a] R.E. = relative error.

ly retained on C2 and not separated from endogenous substances. Yamashita et al. [23] reported that peak enrichment could be attained at the top of the second column following ion-pair chromatography on the 1st column. TBAB is generally used as an ion-pair reagent to analyze acidic compounds. When TBAB was equilibrated in an ODS column, tetra-*n*-butylammonium ions (TBA) were strongly adsorbed onto the ODS. Under the conditions used, TBA could not be washed from the column when only a small volume of the mobile phase, containing no TBAB, was introduced by heart-cutting, and acidic compounds would be strongly retained on the column by ion-pair formation. Thus, enrichment of the acidic compounds at the top of C2 would be anticipated.

Table 3
Regression parameters for the validation of MTX in monkey plasma[a]

Day	Slope	Intercept	Correlation coefficient
1	14	9	0.99997
2	14	7	0.99999
3	13	8	1.00000

[a] Weighted linear regression: 1/C.
Concentration range: 5–1000 ng/ml.

As shown in Fig. 3, when a fresh Guard-pak μBondapak C_{18} column was used as C1, comparable peak heights for MTX were obtained with and without column switching at a concentration of TBAB higher than 10 m*M*. This is explained by the enrichment of MTX at the top of C2. Comparable peak heights were also obtained at 15 m*M* TBAB after 30 repeated injections of 100-μl aliquots of monkey plasma to the Guard-pak μBondapak C_{18} column. At a TBAB concentration higher than 10 m*M*, deterioration of the column efficiency in C1 after repeated plasma injection was negligible. In this condition, the acetonitrile concentration in the mobile phase used for elution of MTX from C2 (MP3, acetonitrile concentration, 18%) was much higher than that used for the introduction of MTX from C1 to C2 (the mixture of MP1 and MP2, acetonitrile concentration, 6%), so the peak compression obtained might be induced by the gradient effect caused by the difference in

acetonitrile concentration. This possibility, however, could be ruled out for the following reasons. If the peak compression were due to the gradient effect only, it should also be observed when MP3 containing no TBAB was used, as the acetonitrile content in MP3 with and without TBAB were adjusted to give almost the same retention times. As shown in Fig. 3, however, peak compression was insufficient without TBAB.

Thus, the influence of the deterioration in efficiency of C1 was minimized by using ion-pair chromatography with TBAB in C2, which made application of on-line solid-phase extraction of MTX to routine analysis possible.

The established procedure was applied to a pharmacokinetic study of MTX in monkey. A typical plasma concentration–time curve after i.v. administration of MTX (0.5 mg/kg) is shown in Fig. 5. The plasma concentration of MTX 120 min after administration (7 ng/ml) was accurately determined and the elimination phase of plasma MTX was successfully followed. Thus, the proposed method could be used for the pharmacokinetic study of MTX in monkey, especially at low dose. This method would be applicable to the clinical study of MTX.

5. Conclusions

In order to develop a sensitive and simple method for the determination of plasma MTX, on-line solid-phase extraction following direct sample injection was investigated. By using ion-pair chromatography with TBAB in C2, MTX seemed to be enriched at the top of C2 after heart-cutting, and the deterioration in efficiency of C1 induced by direct injection of plasma was minimized. The method was suitable for pharmacokinetic study of low-dose MTX in monkey.

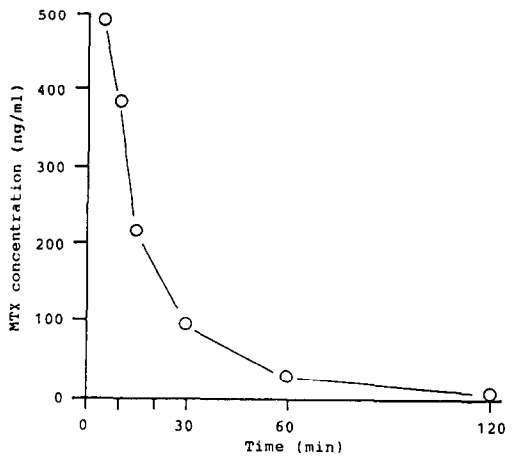

Fig. 5. Plasma concentrations of MTX after i.v. administration of MTX (0.5 mg/kg) to a monkey.

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